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ANALYSIS OF PHENELZINE, LIDOCAINE AND DOXAPRAM: APPLICATION TO THE STUDY OF THEIR METABOLISM AND PHARMACOKINETICS

by

© GEORGE TOROK-BOTH

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY
IN
PHARMACEUTICAL SCIENCES

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA
Spring 1987

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ANALYSIS OF PHENELZINE, LIDOCAINE AND DOXAPRAM: APPLICATION TO THE STUDY OF THEIR METABOLISM AND PHARMACOKINETICS submitted by GEORGE TOROK-BOTH in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in PHARMACEUTICAL SCIENCES.

R T Coutts

Supervisor

Glen B. Baker

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External Examiner

Date January 7th 1987

This thesis is dedicated to my wife

Rita

ABSTRACT

Several analytical procedures were developed for the analysis of phenelzine, lidocaine and doxapram and their respective metabolites. The procedures developed allowed the low level detection of these compounds in a variety of biological fluids and permitted identification of their metabolites by interpreting their electron-impact and chemical ionization mass spectra. Once developed these procedures were utilized in the study of the metabolism and pharmacokinetics of phenelzine, lidocaine and doxapram.

When phenelzine was administered to rats for several days, the conversion of phenelzine to neutral and basic metabolites appears to increase while the conversion of phenelzine to acidic or unextractable metabolites decreased. Ethylbenzene, one of the neutral metabolites of phenelzine identified in this study, increased almost 4-fold in response to chronic phenelzine treatment. Whether this was due to an increase in the formation of 2-phenylethylamine or to a decrease in its elimination could not be determined from these studies. In addition to being metabolized, phenelzine was also found to be eliminated in the bile of rats, either free or as an acid labile conjugate.

Several new metabolites of lidocaine were identified in the urine of rats administered this drug. The presence of these metabolites suggests that the metabolism of lidocaine is far more complicated than first realized. Although these metabolites were not observed in the urine of humans, another metabolite, 4-hydroxymonoethylglycinexylidide

was detected in the urine of a patient administered lidocaine.

4-Hydroxy-2,6-xylidine, a major urinary metabolite of lidocaine in humans, was shown to be a true metabolic product and not an artefact of the analytical procedure. The glucuronide conjugate of 4-hydroxy-2,6-xylidine could be isolated intact from the urine of patients treated with lidocaine.

Doxapram is currently being evaluated in premature infants as a respiratory stimulant. In these infants it was observed that the clearance of doxapram was reduced and the half-life was increased, compared to adults, when this drug was infused at a rate of 2.0-2.5 mg/kg/ml. In addition to doxapram, the metabolite 1-ethyl-4-(hydroxyethylamino-ethyl)-3,3-diphenyl-2-pyrrolidinone was also observed in the urine of these infants. The metabolite 1-ethyl-4-(2-morpholinone)-ethyl-3,3-diphenyl-2-pyrrolidinone could not be detected in either urine or plasma.

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List of Abbreviations

AUC	Area under the blood concentration-time curve
BA	Benzoic acid
CNS	Central Nervous System
CI	Chemical Ionization
CI/MS	Chemical Ionization-Mass Spectrometry
CYT P450	Cytochrome P450
DOX	Doxapram
ECD	Electron Capture Detector
EI	Electron-Impact
EI/MS	Electron Impact-Mass Spectrometry
EBZ	Ethylbenzene
d_{10}-EBZ	Decadeuterated-Ethylbenzene
FID	Flame Ionization Detector
FAD	Flavin-Adenine Dinucleotide
GC	Gas Chromatography
GC/MS	Gas Chromatography-Mass Spectrometry
GX	Glycinexylidide
T1/2	Half-life
HFBA	Heptafluorobutyric Anhydride
HPLC	High Performance-Liquid Chromatography
HRMS	High Resolution-Mass Spectrometry
h	Hour(s)
4-OH-GX	4-Hydroxyglycinexylidide
3-OH-LID	3-Hydroxylidocaine
3-OH-MEGX	3-Hydroxymonoethylglycinexylidide
m-OH-PAA	m-Hydroxyphenylacetic Acid
p-OH-PAA	p-Hydroxyphenylacetic Acid
4-OH-Xyl	4-Hydroxy-2,6-xylidine
i.p.	Intraperitoneal
i.v.	Intravenous
Kg	Kilogram

LID	Lidocaine
MS	Mass Spectrometry
ug	Microgram
ul	Microliter
mg	Milligram
ml	Milliliter
min	Minutes
MAO	Monoamine Oxidase
MAOI	Monoamine Oxidase Inhibitor
MEGX	Monoethylglycinexylidide
ng	Nanogram
NADPH	Nicotinamide-Adenine Dinucleotide Phosphate
NPD	Nitrogen-Phosphorus Detector
PFBA	Pentafluorobenzyl Alcohol
PFBC	Pentafluorobenzoyl Chloride
PFPA	Pentafluoropropionic Anhydride
PLZ	Phenelzine
d_4-PLZ	Tetradeuterated-Phenelzine
PAA	Phenylacetic Acid
d_2-PAA	Dideuterated-Phenylacetic Acid
d_4-PEA	Tetradeuterated-2-Phenylethylamine
PEA	2-Phenylethylamine
PBS	Phosphate Buffered-Saline
Cl_r	Renal Clearance
SIM	Selective Ion Monitoring
TLC	Thin Layer Chromatography
Cl_{TB}	Total Body Clearance
TFAA	Trifluoroacetic Acid
U.V.	Ultraviolet Light
UDPGA	Uridine Diphosphate- -Glucuronic Acid
Xyl	2,6-Xylidine, 2,6-dimethylaniline
α	Disposition Constant for the distributive phase in a two compartment-open model
β	Disposition constant for the elimination phase of a two compartment-open model

Preface

Investigations were conducted on the metabolism and pharmacokinetics of three drugs phenelzine (PLZ), lidocaine (LID) and doxapram (DOX), which undergo extensive metabolism in the body. However, insufficient information is available on their *in vivo* and *in vitro* metabolism or the metabolites which are subsequently formed. The investigations described in this thesis were conducted to provide a more complete understanding of their metabolism and pharmacokinetics.

The lack of information regarding the metabolism of these drugs is primarily due to the absence of appropriate analytical techniques available for their analysis. All three drugs, and their metabolites, are present in biological fluids at low concentrations, yet few procedures have been developed for their low level detection. Emphasis in this thesis was placed initially on developing efficient extraction, separation and detection methods for the analysis of these compounds. The methods of analysis chosen for these studies were gas-chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) which provided sensitive analytical procedures and allowed unequivocal identification of the parent drugs and metabolites isolated from biological samples. Once developed, these procedures were used to investigate the metabolism and pharmacokinetic studies of these drugs.

All three drugs are used extensively in the clinical situation. Where available (for the study of LID and DOX), samples from patients receiving these drugs were obtained for these investigations. For the study of PLZ metabolism and pharmacokinetics a rat animal model was used, rather than using samples obtained from humans. Much of the previous work on PLZ metabolism, conducted by other investigators, has been performed using this animal model. It is hoped that results obtained in the animal model will give insight into the metabolism of PLZ in man.

The introduction to follow is intended to cover pertinent aspects of the pharmacology, pharmacokinetics and metabolism of the drugs of interest. Current methods of analysis will also be described.

1. Introduction

1.1 Pharmacology

The observation that tuberculous patients treated with isoniazid developed a feeling of well being resulted in the application of this monoamine oxidase inhibitor (MAOI) to the treatment of depression. The acute toxicity associated with isoniazid limited its use for the treatment of this disorder. Since its discovery, however, various other MAOIs have been developed. Currently only three MAOIs, tranylcypromine 1, PLZ 2[1] and isocarboxazid are marketed in Canada as antidepressants. Pargyline, a compound initially developed as a selective MAOI, is marketed as an antihypertensive. Of the three MAOIs available, PLZ is the most frequently prescribed.

This ability to inhibit monoamine oxidase (MAO), the enzyme responsible for the oxidative deamination of adrenergic, serotonergic and dopaminergic neurotransmitters and several "trace amines", leads to substantial increases in their concentrations within the central nervous system (CNS) (1). Increases in these neurotransmitters, much like amphetamine stimulation, generates CNS excitability and elevates mood.

MAO exists in two distinct isozyme forms (MAO A and B) each having different substrate specificities and inhibition properties. MAO A preferentially catalyzes the conversion of norepinephrine and 5-hydroxytryptamine to their respective alcohol or acid metabolites, while MAO B converts 2-phenylethylamine (PEA) and the exogenous substrate benzylamine to their respective deaminated products (1). PLZ, tranylcypromine and isocarboxazid are all non-specific inhibitors of both MAO isozymes.

The oxidative deamination of the biogenic amines by MAO is believed to be initiated by the formation of an imine (2). The reaction requires the cofactor flavin-adenine dinucleotide (FAD) (Fig. 1). MAO differs from other amine oxidases in its ability to act on secondary, as well as tertiary amines (1).

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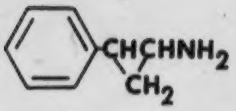
[1] The nitrogens of phenelzine are designated according to the IUPAC method, $C_6H_5CH_2CH_2NHN'H_2$.



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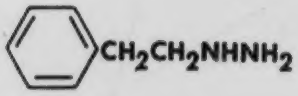

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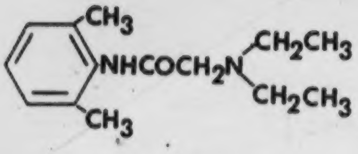

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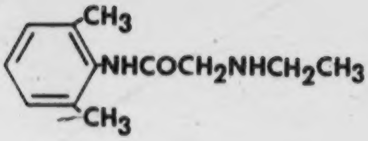

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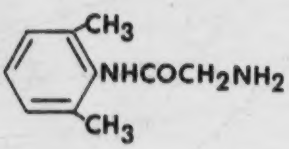

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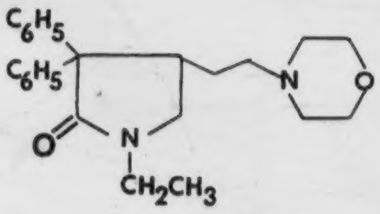

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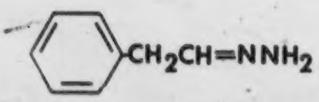

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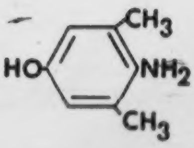

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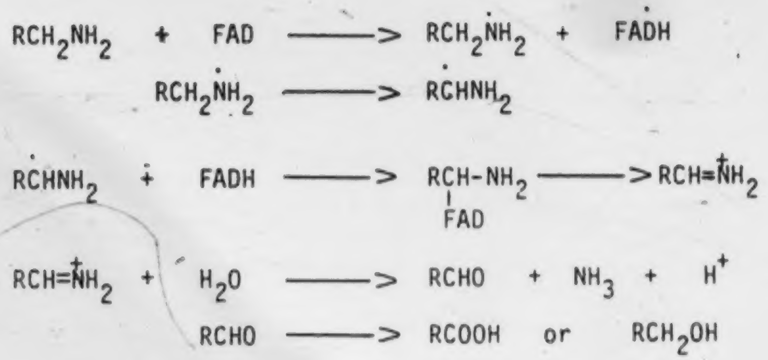


$$\mathrm{RCH_2NH_2} + \mathrm{FAD} \longrightarrow \mathrm{RCH_2\dot{N}H_2} + \mathrm{\dot{F}ADH}$$

$$\mathrm{RCH_2\dot{N}H_2} \longrightarrow \mathrm{R\dot{C}HNH_2}$$

$$\mathrm{R\dot{C}HNH_2} + \mathrm{FADH} \longrightarrow \underset{\mathrm{FAD}}{\mathrm{RCH}}\mathrm{-NH_2} \longrightarrow \mathrm{RCH{=}\overset{+}{N}H_2}$$

$$\mathrm{RCH{=}\overset{+}{N}H_2} + \mathrm{H_2O} \longrightarrow \mathrm{RCHO} + \mathrm{NH_3} + \mathrm{H^+}$$

$$\mathrm{RCHO} \longrightarrow \mathrm{RCOOH} \text{ or } \mathrm{RCH_2OH}$$

Figure 1. The proposed mechanism for the oxidative deamination of primary amines.

PLZ is a unique MAOI since not only does it inhibit MAO A and B (3), but it also serves as a substrate for these enzymes (4,5,6). The mechanism by which PLZ inhibits MAO has yet to be established. It has been observed, though, that when MAO is inhibited by this MAOI, PLZ can no longer be detected (7). Tetradeuterated PLZ (d_4-PLZ), in which deuterium atoms were incorporated into the side chain, has a reduced potential for oxidation (7,8), yet the level of MAO inhibition is significantly increased over that produced by an equimolar dose of unlabelled PLZ.

PLZ not only inhibits the oxidative deamination of various amines, but also effectively inhibits the enzymes responsible for the O-demethylation of p-nitroanisole (9), N-demethylation of N,N-dimethylaniline (10), and aminopyrine (11), and hydroxylation of aniline (12). All these reactions are mediated by cytochrome P450 (CYT P450). Tranylcypromine also effectively inhibits these same metabolic reactions. A similar loss of CYT P450 activity was observed when benzylhydrazine was incubated with hepatic microsomes (13).

Following the administration of PLZ, the level of 5-hydroxytryptamine in rat hypothalamus increased (14). Increases in the levels of dopamine, m- and p-tyramine, and tryptamine were also observed following PLZ or tranylcypromine administration to rats (15). However, only tranylcypromine increased the CNS level of PEA after an acute dose (16). The levels of PEA did not increase following an acute dose of PLZ unless an excessively high dose (50-100 mg/kg) of this MAOI was administered (17). When PLZ at a substantially lower dose (15 mg/kg) was administered daily to rats for prolonged periods, brain levels of PEA did not increase above control values until day 8. By day 19 greatly increased quantities of PEA were detected within the CNS (16).

In the clinical situation, maximal MAO inhibition is achieved within the 1st week of treatment, while antidepressant activity does not appear for 2 or 3 weeks after the start of therapy (18). This finding has led many investigators to suggest alternative biochemical mechanisms of depression. Some investigators believe that antidepressant effectiveness is associated with the desensitization of central α_2-receptors (19), which occurs slowly. Changes in the receptor sensitivity are probably caused by the increase in the level of the catecholamine neurotransmitters as a consequence of MAO inhibition. Recent studies have demonstrated that platelet MAO activity must be at least 80% inhibited before a clinical response is observed (20). When a group of patients were treated with PLZ (60 mg/kg) it was observed that platelet MAO inhibition varied between 59% and 98%. Of those patients with greater than 90% inhibition, 60% improved with the drug therapy, whereas only 40% of those patients with less than 90% MAO inhibition improved (21). This evidence although supportive, does not conclusively establish that significant MAO inhibition is required for antidepressant therapy to be effective.

Unlike PLZ, LID 3 exerts its primary pharmacological effect in the periphery. LID is a local anesthetic which is frequently used to prevent ventricular fibrillation associated with myocardial infarctions (22). The primary mechanism of action of LID is to reduce the action potential duration of the fast sodium channels. It is the ionic form of LID which blocks these tetrodotoxin-sensitive sodium channels (23). Not only is LID effective, but its metabolites monoethylglycinexylidide (MEGX) 4 and glycinexylidide (GX) 5 can also block these sodium channels. From animal studies it was found that MEGX was 83% as potent as LID, while GX had only 10% of the LID activity (24).

Toxic side effects have also been observed with all three compounds. These side effects can be quite serious and include convulsion, hypotension, respiratory arrest and circulatory collapse (25). Side effects were observed even when the blood concentration of LID was within the therapeutic range. When this occurs the side effects are believed to be due to elevated levels of MEGX and GX (26,27).

Unlike the previous two drugs (PLZ and LID), the site of action of DOX 6 is both in the periphery and CNS. This drug is frequently used as a respiratory stimulant in patients suffering from respiratory failure or during recovery from local anesthetics (28). DOX acts on the carotid body chemoreceptors, found in the periphery, and the respiratory neurons located in the brain stem (29,30).

Recently, DOX has been administered to neonates born to mothers who received either narcotic analgesics or general anesthetics during labor (31,32). The successful application of this drug to these infants has led to its use for the treatment of recurrent apnoea in premature infants (33,34). Currently, theophylline is the drug of choice to treat this disorder, however, many infants are therapeutically unresponsive to this drug.

DOX has a wide margin of safety and few side effects have been observed in infants. Side effects which are known to occur include agitation, excessive crying, disturbed sleep, and erratic limb movement (35). Both gastrointestinal irritation and liver dysfunction have also been observed. More recently it has been demonstrated that the administration of DOX to premature infants can cause an increase in blood pressure (36). This potentially serious side effect, however, may be avoided with proper dosing in these patients. Currently infants are administered an equivalent adult dose, which has been adjusted to the weight of the infant (37). In order to determine proper infant dosing both the therapeutic drug level and pharmacokinetics of DOX must be established in these infants.

DOX also causes marked inhibition of microsomal enzymes, much like the CYT P450 inhibitor SKF-525A. Decreases in the activities of aminopyrine-N-demethylase, aniline hydroxylase and phenobarbital oxidase were observed in mice following the administration of DOX (38).

1.2 Pharmacokinetics

Determining the rate at which a drug is eliminated from the body is not only essential to establish the frequency of drug administration but is also necessary to determine whether a drug is eliminated by zero order or first order kinetics. Drugs which are eliminated by Michaelis-Menten kinetics pose special problems since it is difficult to predict their steady state blood levels (39). PLZ, LID and DOX are all potential candidates for saturable elimination pathways, since these drugs are extensively metabolized. Of the three drugs, only LID, whose dose- and time-dependant kinetics have been previously demonstrated, is therapeutically monitored.

1.2.1 Elimination

Recent studies indicate that PLZ, LID, and DOX all undergo significant first pass metabolism following an oral dose. Comparisons of areas under the blood concentration-time curve (AUC) following an oral and intravenous dose (i.v.) have shown that the oral bioavailability of LID, PLZ and DOX was 35%, 16.7% and 60.8%, respectively (22,35,40). Both LID and DOX are administered by i.v. infusion, a route which reduces the significance of first pass elimination. Administration of LID by this route removes the incidence of CNS and gastrointestinal side effects frequently observed when large oral doses of LID are given. The increased incidence of side effects, following oral administration, is believed to be due to the increased production of MEGX and GX (22). PLZ, on the other hand, is currently administered orally. Peak blood levels of this drug were found to be only 1-10 ng/ml following a 60 mg/kg dose of PLZ (41). The rapid elimination of PLZ, LID and DOX is reflected by their short biological half-lives. From single dose studies in man, half-lives were found to be 0.87, 1.0, 3.4h, respectively (28,42,43). Less than 1% of an oral dose of LID and PLZ (43,44) and 5.4% of a dose of DOX (45) are eliminated by renal excretion as the parent drug.

Since these drugs are eliminated almost entirely as metabolites, saturation of their metabolism may occur influencing the kinetics of these compounds. LID and DOX are both administered by i.v. infusion, at doses which could saturate the enzymes responsible for their elimination. PLZ, although administered at low doses, is known to inhibit its own metabolism (4).

Metabolic pathways may become saturated not only by limited capacity but also by substrate or product inhibition of the enzyme (39). Two examples of substrate or product inhibition are propranolol (46) or the phenytoin metabolite, 5-(p-hydroxyphenyl)-5-phenylhydantoin (39), respectively. Propranolol inhibits its own metabolism and after prolonged administration will cause changes in its own elimination. 5-(p-Hydroxyphenyl)-

5-phenylhydantoin, on the other hand, inhibits the metabolism of phenytoin.

For drugs which demonstrate saturation kinetics, drug clearance is concentration dependent and the liver extraction ratios will decrease with increasing concentrations of these drugs (47). These drugs should be therapeutically monitored since it is difficult to accurately predict their plasma levels (48).

Upon chronic administration for 13 days, the half-life of PLZ increased from 0.87 to 3.11 h. In addition, the amount of PLZ excreted in the urine rose from 1% to almost 5% of the administered dose (42). The elimination of LID was also found to be dose- and time-dependant. Accumulation of LID was observed in people who received infusions of this drug for prolonged periods (greater than 24 h) (49). In these people, a true steady state was never attained. This inability to achieve a steady state was reflected by a significant decrease in the hepatic extraction ratio of LID (50). Product inhibition is believed to be partly responsible for the observed saturation kinetics. MEGX was found to inhibit, *in vitro*, the N-dealkylation pathway of LID (51). However, more recently evidence has suggested, at least in rats, that it is not the dealkylation pathway but rather the hydroxylation pathway which could be saturated (52,53).

The elimination of DOX, in adults, is linear. However, administration of this drug to premature infants requires further pharmacokinetic evaluation. In general, infants have a reduced capacity to metabolize drugs (54). At term CYT P450 and nicotinamide adenine dinucleotide-cytochrome c reductase levels approach half the adult values. Infants have an observable reduction in p-hydroxylation while dealkylation efficiency remains comparable to that of adults. Both sulfate and glycine conjugation are also comparable to adults but glucuronidation is reduced and only reaches adult values by 3 years of age (54).

1.2.2 Biliary Excretion

Biliary excretion is a mechanism, much like urinary excretion, by which drugs and their metabolites are eliminated from the body. The excreted drug, however, can be readsorbed from the intestine back into the portal vein circulation.

Several factors can influence the biliary excretion of a compound (55). The presence of a strongly polar group or potentially ionizable moiety augments biliary excretion. Brauer (56) noted that substances normally excreted into bile are usually organic carboxylic acids with molecular weights greater than 300. This threshold molecular weight varies among species. In the guinea pig, rabbit, and man these values are 400, 475 and 500, respectively (57). Terbutaline (MW=225), which is extensively excreted into the bile of rats, undergoes negligible biliary excretion in dogs and man (58). Comparison of the biliary excretion of quaternary ammonium cations in the bile of rabbits, rat and guinea pigs indicated that molecular weight may not be important in organic cation excretion (59).

Biliary excretion often involves compounds which are conjugated. Conjugation increases both the polarity and the size of the molecule. However, biliary excretion of unconjugated drugs has also been observed. Compounds such as chlorothiazide (60), acetaminophen (61) and oxazepam (62) can be isolated in bile unconjugated.

The rate of bile flow or drug conjugation can influence biliary excretion. Bile flow is independent of the hepatic blood flow once a critical blood pressure has been attained (63,64). In contrast, gastrointestinal hormones (65-67) and feeding (68) can stimulate bile flow. Several drugs (e.g. valproic acid, naltrexone and probenecid) can also stimulate bile flow (69-71). Bile flow rate may also vary with circadian rhythm; in rats, peak flow rates were observed at midnight (72). Both bile flow and biliary excretion of a drug can be reduced by a number of factors. In newborn rats, dogs, rabbits and guinea pigs, biliary excretion is not fully developed (73,74). Decreases in bile flow and biliary excretion of bilirubin and bromosulfophthalein resulted from experimental hypothermia induced by the administration of anesthetic agents (75). Acute treatment of rats with the hepatotoxin carbon tetrachloride significantly reduced the biliary clearance of bromosulfophthalein (76). The excretion of acetominophen into bile was also reduced in rats anesthetized with diethyl ether (77), a result associated with the effect of the anesthetic on the concentration of uridine diphosphate-α-glucuronic acid (UDPGA) in the liver (76-80).

Enterohepatic recirculation is an important process in the conservation of a variety of endogenous compounds excreted in the bile. Numerous drugs and xenobiotics, such as norethisterone (81), propachlor (82), diclofenac (83), morphine (84), diphenylacetic acid (84) and diphenylhydantoin (85) also undergo enterohepatic recirculation.

Enterohepatic recirculation obviously influences the kinetics of drug elimination. This process significantly increases the apparent volume of distribution and prolongs the elimination half-life of a drug (86). Factors which influence biliary excretion and thus enterohepatic recirculation can therefore affect the overall kinetics of a drug.

The extent to which a drug undergoes biliary excretion is highly dependent on the route of administration. Following bile duct cannulation, which prevents normal bile flow into the intestine, the AUC of isotretinoin was most significantly reduced following oral administration. The smallest difference in the AUC between intact animals and bile duct-cannulated animals was observed following i.v. administration of the drug (87). A similar observation was made when scopolamine-N-butyl bromide was administered either intraportally or into the femoral artery of rats (88). Several PLZ-like compounds, pivhydrazine (21%) and mebanazine (24%) and benzylhydrazine (3%), are eliminated in bile as conjugates to the extent indicated. These conjugates were tentatively identified as N-glucuronides since they could be easily hydrolyzed by β-glucuronidase or in weakly acidic solutions (pH 5.5) (89). The possibility that a hydrazone conjugate, rather than an N-glucuronide, was excreted in the bile was not considered by these investigators. ~~Hydrazones are them~~selves easily hydrolyzed in weakly acidic solutions (90). Low fecal radioactivity levels following the administration of radiolabelled pivhydrazine or mebanazine indicates that significant readsorption of these compounds occurs from the intestine (89).

Approximately 30% of a dose of LID was observed in a 24 h rat bile sample. The metabolites present were the glucuronide conjugates of 3-hydroxy-lidocaine (3-OH-LID) and 3-hydroxy-monoethylglycinylxylidide (3-OH-MEGX) (51). The absence of these metabolites in feces suggests that these two compounds undergo enterohepatic recirculation.

Similar results were obtained when DOX was administered to animals. Significant amounts of DOX material were observed in the bile of treated dogs (91). The material isolated in bile contained three unidentified DOX metabolites. In a second study, evidence was obtained which indicated that DOX itself was also eliminated in bile (40). An increase in the plasma concentration of DOX 4 h after the adsorption phase following oral administration of this drug, implied that biliary excretion and subsequent readsorption of DOX had occurred (40).

1.3 Metabolism

Following administration, a drug encounters a variety of enzymes capable of converting the drug to other forms, which are usually pharmacologically inactive. Drug metabolism occurs in various tissues, including the gut wall, liver, lung, kidney, and brain (92). The liver is considered the major site of drug metabolism, but other organs can contribute significantly, since they may receive greater cardiac output and thus more of the drug. The gut wall, liver and lung all contribute to the first pass metabolism of an orally administered drug.

All metabolic reactions involving exogenous substrates are classified as either phase 1 or phase 2 metabolic conversions (93). Phase 1 reactions irreversibly alter the substrate. Examples of these types of reactions include oxidation, reduction, and hydrolysis. Phase 2 conversions result in the addition of an endogenous constituent to the substrate. These reactions usually yield products which are pharmacologically inactive and much more water-soluble than the parent molecule (93).

1.3.1 Phase 1 Metabolism

Oxidations at carbon, nitrogen and sulfur atoms are frequently encountered in drug metabolism. These reactions are mediated by CYT P450, an enzyme widely distributed throughout the body (94).

Clineschmidt and Horita (4,95) and others (96) have demonstrated that PLZ is oxidized to phenylacetic acid (PAA), both *in vivo* and using rat liver homogenates. PAA is almost completely conjugated with glutamine prior to its excretion into urine (97). This conversion of PLZ to PAA, *in vivo*, was confirmed using d_4-PLZ (98). When this compound was administered to rats, dideuterated PAA (d_2-PAA) could be isolated from both the urine and tissue. Benzylhydrazine is similarly oxidized metabolically to benzoic acid (BA) (99,100), which is subsequently excreted in urine as hippuric acid (99).

When PLZ was incubated with purified MAO it was dehydrogenated to phenylacetaldehyde hydrazone 7. The enzymatic conversion of phenylhydrazine to phenyldiazene led to the inactivation of CYT P450 (101). Similar observations were made with D,L-(1-methyl-2-phenyl)ethylhydrazine, suggesting that enzyme inhibition, particularly of MAO, may involve a diazene intermediate (101). The mechanism by which phenylacetaldehyde hydrazone is formed is not understood. PLZ may initially be converted to a diazene which in turn undergoes intramolecular rearrangement to form the corresponding hydrazone (101). It is likely that MAO is inhibited by the diazene intermediate. The resulting hydrazone could, either enzymatically or chemically, be converted to PAA.

Diazene formation may initially involve microsomal N-oxidation of the hydrazine to an N-hydroxy intermediate which dehydrates to the diazene (Fig. 2) (102). Metabolic N-oxidation, like oxidative deamination, requires FAD rather than nicotinamide adenine dinucleotide as a cofactor (93). Whether diazenes can rearrange to form phenylacetaldehyde hydrazone 7 or it is generated in a manner analogous to deamination of primary amines by MAO cannot be established from our current knowledge. The possibility that phenylacetaldehyde hydrazone is formed in a manner analogous to the MAO-mediated deamination of primary amines cannot be discounted.

Considerable amounts of amphetamine have been isolated from rat brain after the administration of pheniprazine (103). The formation of amphetamine involves the fission of the N-N bond of pheniprazine. A similar mechanism has been used to explain the conversion of

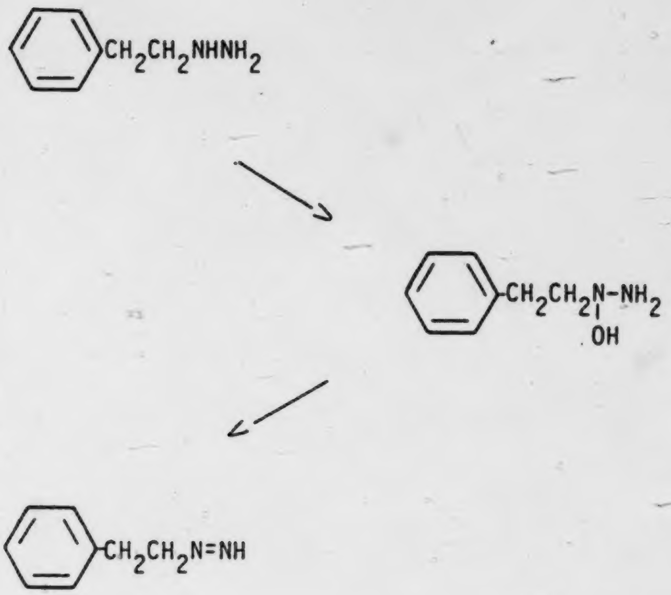


Figure 2. The proposed metabolic pathway for the conversion of phenelzine to its corresponding diazene metabolite. The reaction is believed to proceed through a metabolic N-oxidation.

PLZ to PEA. Tetradeuterated PEA (d4-PEA) was isolated from tissue of rats administered d_4-PLZ (98). The formation of amphetamine and PEA may both involve N-oxidation prior to cleavage of the N-N bond.

The metabolic formation of alkanes from substituted hydrazines is unique to this class of compounds. Ethylbenzene (EBZ) has been identified as an *in vitro* metabolite of PLZ (13). Phenylhydrazine is also readily metabolized to nitrogen and benzene. This reaction could be catalyzed by oxyhemoglobin (104) or catalase (105). Incubation of monoacetylphenylhydrazine, acetylated on the primary nitrogen, with oxyhemoglobin also produced benzene and nitrogen (106). When ^{14}C-iproniazid, labelled at the C-2 carbon of the isopropyl group, was administered to rats, 9% of the dose was expired as ^{14}C-propane (107). Prough et al (108), using liver homogenates demonstrated the conversion of several simple monoalkylhydrazines to their respective hydrocarbons. Substrates investigated were methyl- , ethyl-, n-propyl-, and n-butylhydrazine. Using the radical scavenger α-pyridyl-1-oxide-N-tert-butylnitrone (PBN), free radical adducts of isoniazid and iproniazid were detected when these compounds were incubated with isolated rat liver hepatocytes (109). The metabolic formation of alkanes is believed to occur by a free radical mechanism. CYT P450 is thought to be involved in these reactions since the electron spin resonance signal, used to detect free radicals, decreased when these enzymes were inhibited (109). However, the conversion of alkylhydrazines to alkanes was not influenced by prior treatment with phenobarbital or 3-methylcholanthrene (110), two common CYT P450 inducers.

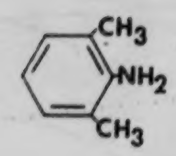
EBZ is converted, *in vivo*, to methylphenylcarbinol, which is further oxidized to mandelic acid (111). Intermediates in the reaction sequence are acetophenone and *omega*-hydroxyacetophenone. This latter compound is the precursor to mandelic acid, a metabolite which accounts for only 1-2% of a dose of EBZ. Other minor metabolites of EBZ are 1-phenylethanol, 2-phenylethanol and PAA (112).

Much like PLZ, LID undergoes extensive metabolism in the body. The primary site of LID metabolism is in the liver, where the enzymatic conversions are mediated by CYT P450

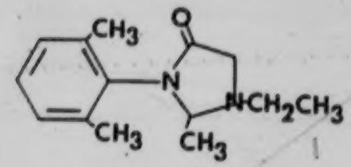
(113). From the structure of LID it is evident that there are three metabolic reactions possible. These are oxidation of the aromatic ring, N-deethylation and amide hydrolysis. Only amide hydrolysis is a non-oxidative reaction, proceeding readily even under anaerobic conditions (114). Following the administration of LID to rats, 3-OH-LID and MEGX were produced metabolically. These two metabolites were further metabolized to 3-OH-MEGX, by either N-dealkylation or 3-hydroxylation (53). In rats, 3-OH-LID and 3-OH-MEGX accounted for 70% of the administered LID dose (115). Other metabolites recovered from urine were 4-hydroxy-2,6-xylidine (4-OH-Xyl) 8 (12.5%), 2,6-xylidine (Xyl, 2,6-dimethylaniline) 9 (1.5%), MEGX (0.7%) and GX (2.1%). Only 0.2% of unchanged LID was eliminated in the urine.

Species differences have been observed in the metabolism of LID. In man, the major urinary metabolite is 4-OH-Xyl, while 3-OH-LID and 3-OH-MEGX were minor metabolites. In contrast, the amounts of unchanged LID, MEGX, GX and Xyl recovered from the urine of humans administered LID were equivalent to the amounts recovered in rat urine, on a per cent dose basis. Studies conducted on guinea pigs demonstrated that Xyl (16%) was a significant metabolite in this species (115). Age-related differences in the metabolism of LID have also been observed. Neonates excreted larger quantities of LID (20%) and MEGX (20%) than adults; however, the amount of 4-OH-Xyl excreted in their urine was significantly reduced (8%). These infants are believed to have a reduced ability to hydroxylate compounds, while their amidase activity is believed to be equivalent to that of adults (116). The quantity of Xyl formed was equal in both infants and adults. Other minor urinary metabolites which have been detected in human urine are N-hydroxylidocaine, N-hydroxymonoethylglycinexylidide and a cyclized metabolite 10 (117,118). The nitrogen involved in the N-oxidation has not been established. This cyclized metabolite is now believed to be an artefact, formed between MEGX and acetaldehyde present in the extraction solvent (119).

Using rabbit liver microsomes, Hollunger (120) demonstrated that MEGX was actively hydrolyzed by an amidase, present in the preparation, to Xyl and N-ethylglycine. This amidase had little activity towards LID or GX, either in liver microsomes or the purified enzyme


CH3
NH2
CH3

9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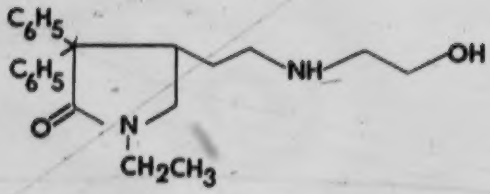
O
N
NCH2CH3
CH3
CH3

10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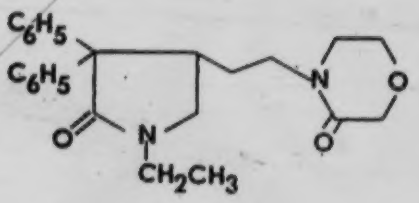
C6H5
NH
OH
O
N
CH2CH3

11


C6H5
C6H5
N
O
O
N
O
CH2CH3

12

preparation (120). Amide hydrolysis of LID or its metabolites appears to be a minor metabolic pathway in rats (115).

The aromatic hydroxylation of LID or MEGX can be saturated even at low substrate concentrations, while N-deethylation has a high substrate capacity. These two enzymes are believed to occupy distinct sites in the liver, each having a different affinity for LID. The low affinity site is believed to be responsible for N-deethylation, a process which is inhibited by MEGX (51).

The human metabolism of DOX, much like PLZ and LID, is primarily by oxidative phase 1 reactions. The major portion of urinary radioactivity following the administration of ^{14}C-labelled DOX to dogs was observed as 1-ethyl-4-(2-hydroxyethyl-aminoethyl)-3,3-diphenyl-2- pyrrolidinone 11. This metabolite accounted for 40% of the urinary radioactivity and has also been observed in the urine of premature infants (45). Other metabolites identified in the urine of dogs are shown in figure 3. In adults, the metabolite 1-ethyl-4-(2-morpholinone)-ethyl-3,3-diphenyl-2 -pyrrolidinone (henceforth referred to as the morpholinone metabolite 12) was found in plasma at concentrations equivalent to unchanged DOX (40,121). In a second study, the primary amine metabolite of DOX (compound b, Fig. 3) was identified and found to represent 25% of the administered dose of DOX (91).

1.3.2 Phase 2 Metabolism

Conjugation of drugs and metabolites constitutes a major mechanism of drug elimination. The term conjugation refers to a number of metabolic reactions in which a xenobiotic is coupled to an endogenous moiety, such as glucuronic acid, glucose, amino acids, acetic acid, glutathione or inorganic sulfate. These endogenous compounds are utilized in the classical conjugation reactions. However, a variety of unusual conjugates, some resulting from reactions with DNA and protein, can occur (122).

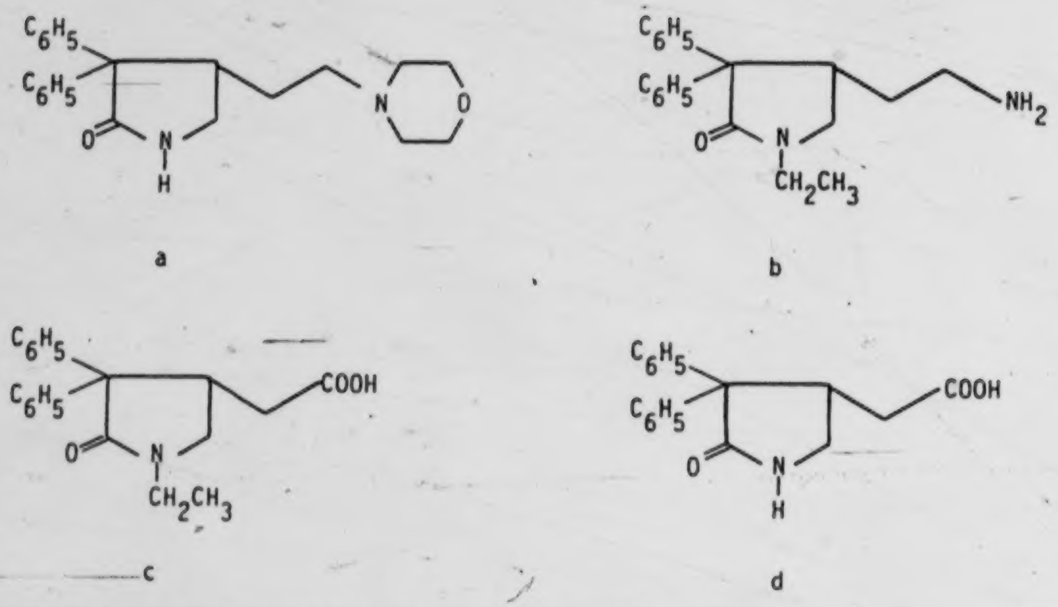


Figure 3. Other metabolites of doxapram isolated in the urine of dogs.
a- 4-(2-morpholinoethyl)-3,3-diphenyl-2-pyrrolidinone
b- 1-ethyl-4-(2-aminoethyl)-3,3-diphenyl-2-pyrrolidinone
c- 1-ethyl-4-(2-acetic acid)-3,3-diphenyl-2-pyrrolidinone
d- 4-(2-acetic acid)-3,3-diphenyl-2-pyrrolidinone.

Conjugates are predominantly inactive pharmacologically, although there are examples of conjugates which do retain pharmacological activity (123,124). For example, N'-acetylphenelzine (ACP) retains MAO inhibiting properties, both *in vivo* and *in vitro* (125). N-Acetylphenelzine is believed to be an *in vivo* conjugate of PLZ (157).

1.3.2.1 Glucuronidation and Sulfation

Glucuronidation and sulfation constitute the most commonly observed conjugating pathways. Glucuronic acid, in its activated form UDPGA, can react with a variety of functionalities (-OH, -COOH, $-NH_2$, $-NR_2$ and -SH) (122). These reactions are catalyzed by the enzyme UDP-glucuronyl transferase. Formation of sulfate conjugates of phenolic, alcoholic and primary amines have also been demonstrated (122). This reaction is catalyzed by the enzyme sulfotransferase, and utilizes sulfate derived from either D,L-cysteine or dietary sources. In order to participate in the reaction, sulfate is converted to an active intermediate, 3'-phospho-adenosine-5'-phosphosulfate (122). Sulfate conjugation can be readily exhausted due to the limited availability of sulfate (126). Since several functional groups can be conjugated to either sulfate or glucuronic acid, a dose-dependent relationship between sulfation and glucuronidation is often observed. At high doses of the drug, glucuronidation will predominate, while the significance of sulfation will increase at lower drug doses.

4-OH-Xyl, the major metabolite of LID in man, is extensively conjugated prior to its excretion in urine (115). The 3-hydroxylated metabolites of LID and MEGX, isolated from rat urine, are also excreted as glucuronide conjugates (115).

N-Glucuronidation of a number of primary (127,128) and tertiary amines (129) is known to occur. The LID metabolite Xyl is believed to be conjugated to glucuronic acid in the guinea pig (115). Neither rats nor men carry out this reaction to any extent. N-Glucuronides of hydrazine drugs have also been indirectly identified. Pivhydrazine and mebanazine are both believed to form N-glucuronides when administered to rats (89). These conjugates were readily hydrolyzed by both β-glucuronidase and weakly acidic conditions.

Following the acid hydrolysis of urine obtained from dogs administered DOX, two conjugated metabolites were tentatively identified. One metabolite was a primary amine while the other was a carboxylic acid (91). Both metabolites were also observed in the bile of these animals.

Sulfate conjugates of PLZ, LID, DOX or their metabolites have not been identified. This is probably a consequence of the difficulty involved in identifying this type of conjugate.

1.3.2.2 Amino Acid Conjugates

Several aromatic and aliphatic acids have been isolated from urine as conjugates of glycine, glutamine, taurine and ornithine. The common acid metabolites, BA and PAA, for example, are extensively conjugated to glycine and glutamine, respectively, before they are excreted in the urine (130,131). BA is a major metabolite of benzylhydrazine and isocarboxazid (99,100). Other compounds identified in urine as their glycine conjugates are salicylic acid (132) and isovaleric acid (133). Isovaleric acid is also excreted as a glutamine or glucuronide conjugate (134).

1.3.2.3 Acetylation

Unlike other conjugation reactions, acetylation yields metabolites which are less polar than the original substrate. This reaction proceeds by the addition of an acetyl group derived from acetyl-coenzyme A (135). This enzyme is present in the cytosol of cells located in the liver, lung, duodenum, thymus, ovary, spleen, uterus, adrenal gland, leukocytes, kidney, bone marrow, salivary gland, pancreas, pineal gland, erythrocytes and brain (136).

Acetylation of a variety of compounds including procainamide (137), dapsone (138), aminoglutethimide (139), nitrazepam and clonazepam (140), isoniazid (141), sulfapyrine (142) and acebutolol (143), have been observed.

Polymorphic acetylation refers to the genetically determined interindividual differences observed in the rate and extent of acetylation. Individuals are referred to as either fast or slow acetylators, with slow acetylation being an autosomal recessive trait (144). In addition to man, polymorphism has been observed in the rhesus monkey (145), den mouse and lab mouse (146) and the Syrian hamster (147). Evidence of polymorphic acetylation in rats is equivocal. Both procainamide and sulfamethazine, two compounds used to determine the rate of acetylation in man (148,149), are monomorphically acetylated in the rat (149,150).

Differences in the rate of acetylation can alter the elimination rate and toxicity of a drug. Fast acetylators usually excrete greater amounts of acetylated metabolite and the half-life (T1/2) of the parent drug is shorter (151). Both the AUC and the steady state concentration of the drug are also lower in fast acetylators (152). Recently, however, it has been demonstrated that these observations may not always be true. Aminoglutethimide was found to have a longer T1/2 and a lower total body clearance (ClTB) in rapid acetylators than in slow ones (139). Caddy et al. (42) found a similar result with PLZ. The T1/2 of PLZ was consistently longer (1.03 and 1.61 h) in two fast acetylators than in three slow acetylators (0.4, 0.58 and 0.72 h).

Acetylation polymorphism is not observed in all tissues within the same animal. Sulfamethazine acetylation in the spleen, kidney and pineal gland were equivalent in both rapid and slow acetylators (153). The differences in acetylation rate in those tissues where it is expressed has been attributed to structural differences rather than differences in the amount of enzyme present (154).

The rate of acetylation of a drug can also be affected by the route of administration. Isoniazid acetylation is the same in both fast and slow acetylators when the drug is administered i.v. (155). It has been suggested that isoniazid acetylation occurs in the small intestine, its site of absorption, and differences in acetylating capacity are expressed there.

The assumption that PLZ undergoes N-acetylation is based on three indirect observations; a) its similarity in structure to isoniazid a drug known to undergo metabolic acetylation (156), b) the ability of highly purified rabbit N-acetyltransferase to metabolize PLZ to N-acetylphenelzine, *in vitro* (96) and c) evidence obtained from clinical studies which suggest that the response to PLZ is slightly greater in people who are slow acetylators (157-159). The purified N-acetyltransferase enzyme is the same enzyme which acetylates isoniazid, sulfamethazine, and procainamide, however both PLZ and monoacetylhydrazine have lower affinities for this enzyme than do these substrates (96). From clinical studies a relationship between acetylator status and MAO inhibition has been suggested (160). Marshall et al. (156) found that slow acetylators respond better to lower doses of PLZ. In this same population an increased incidence of side effects was observed. Other studies have failed to show any relationship between acetylator status and therapeutic effect (161). Slow acetylators excrete twice as much PLZ in urine collected for 24 h than the corresponding fast acetylators (162). These contradictory studies make it difficult to assess the status of PLZ acetylation. One problem in identifying N-acetylphenelzine as a metabolite of PLZ could arise from difficulties encountered in isolating this compound from blood. When a relatively large quantity of N'-acetylphenelzine (100 ug/ml) was added to blood, only 93.6 ug/ml could be isolated at time 0 and only 64.6 ug/ml 15 min later. Prolonged incubation did not further reduce the amount of recoverable N'-acetylphenelzine which remained at 51 ug/ml even after 45 min incubation (125). This suggests the presence, in blood, of saturable sites capable of binding N'-acetylphenelzine irreversibly.

N-Acetylation was not observed when phenylhydrazine, a compound similar to PLZ, was administered (163). In contrast, acetylation does appear to be important in the metabolism of hydrazine itself. Slow acetylators have a higher blood level of hydrazine than do fast acetylators (90). A significant observation was that 23% of a dose of isoniazid could be isolated as N,N'-diacetylhydrazine in fast acetylators while only 4.9% was observed in slow acetylators (164). Acetylhydrazine is a major metabolite of isoniazid (165). In man and rat liver microsomes this metabolite undergoes further oxidation to produce a highly reactive acylating agent (166).

Whether PLZ acetylation occurs has not been fully established. One author, however, has suggested that if acetylation of PLZ does occur it is a minor metabolic pathway (167).

1.3.2.4 Hydrazone Formation

The postulation that PLZ forms hydrazone conjugates with various aldehydes, *in vivo*, has gained increasing support despite the fact that such metabolites have yet to be isolated. Isoniazid, hydralazine and phenylhydrazine all form hydrazones with pyruvic acid and α-ketoglutarate. These hydrazones are believed to be formed non-enzymatically under physiological conditions (110).

Pyridoxal, which can form hydrazones with substituted hydrazines, could conceivably form a conjugate with PLZ. Long term administration of PLZ does result in a vitamin B_6 deficiency (168). Pyridoxine was found to protect mice from lethal doses of PLZ (169). Pyridoxal conjugates of 1,1-dimethylhydrazine are formed, *in vivo* and *in vitro* (170). This conjugation leads to the inhibition of enzymes which require pyridoxal phosphate as a cofactor (171). Chronic treatment of animals with isoniazid resulted in a 12% loss of body weight by the eighth day, an effect which could not be explained by reduced food and/or water intake (172). Poor food utilization has been associated with a vitamin B_6 deficiency (172).

1.4 Analytical Procedures

Analysis of several compounds, with differing chemical and physical properties, are described in this thesis. GC techniques were developed for the analysis of PLZ, LID, DOX, and their metabolites. These included a) metabolites of PLZ: EBZ and PAA b) the LID metabolites: 4- and 3-OH-LID, 4- and 3-OH-MEGX, 4-hydroxyglycinexylidide (4-OH-GX), 4-OH-Xyl and its glucuronide conjugate and c) metabolites of DOX: 1-ethyl-4-(2-hydroxyethylaminoethyl)-3,3-diphenyl-2-pyrolidinone 11 and the morpholinone metabolite 12. In this section currently available methods for the analysis of these compounds are reviewed. Although GC separation was the method of choice in this study, where relevant, other analytical techniques will also be discussed.

1.4.1 Isolation of Compounds from Biological Fluids

1.4.1.1 Bases

For the GC analysis of PLZ, LID and DOX and their basic metabolites, these compounds must initially be isolated from the aqueous sample. Extraction of these compounds into a suitable water-immiscible solvent, following adjustment of the pH to alkali condotions, is commonly used (173). The solvent used for extraction must have a low boiling point, not be prone to emulsion formation, and the partition coefficient of the compound must favor the solvent (large Kp).

Caddy et al. (162) extracted PLZ from a basic solution (2M NaOH) into diethyl ether. GC analysis of this extract demonstrated the presence of several products. From this it was concluded that PLZ is unstable in basic solutions. To overcome this problem several investigators (41,174,175) extracted PLZ from a blood sample buffered to pH 6.8. The extraction efficiency of this procedure was not reported.

The most frequently used method for the isolation of PEA from urine (176), homogenized brain (177,178) and liver tissue (177) is again by simple organic solvent extraction. Martin et al (179) used Amberlite®15 adsorption columns to isolate PEA from brain tissue. The absorbed PEA was eluted with methanolic/HCl. The ion pairing agent di-(2-ethylhexyl)phosphoric acid has also been tried to enhance the extraction of PEA from tissue samples (180,181). Optimum pH for extraction was between pH 6 and 7 (180). This reagent, however, interfered with the GC procedure. Currently di-(2-ethylhexyl)phosphoric acid is used during sample preparation as a clean-up step

(182).

The most common method of isolating LID and its three basic metabolites (MEGX, GX, Xyl) from blood or urine is by simple solvent extraction from an alkaline solution (115,183-188). The extractibility of these compounds from basic aqueous solutions appears to be efficient. The limit of detection of LID was estimated to be 40 ng/ml when a 1 ml plasma sample was extracted (189).

Much like PLZ and LID, DOX and its basic metabolites can be easily extracted from aqueous samples after adjustment to basic pH. Robson and Prescott (40) isolated DOX and its morpholinone metabolite 12 from 2 ml of plasma by extracting the basified plasma sample with dichloromethane. No additional clean-up step was required prior to the extraction of DOX. Unlike the case with plasma, analysis of DOX in urine required an additional clean-up step to remove endogenous urinary constituents which interfered with the assay (45). Various basic metabolites of DOX were also isolated from urine by initially adsorbing these compounds to a XAD-2 adsorption column (121). The samples were further purified by passing the XAD-2 treated sample through an Aminex® A-5 cation exchange resin.

1.4.1.2 Acidic Compounds

In these studies, the analysis of PAA in biological samples was investigated. In addition, the procedure developed was also used to analyze for BA, m- and p-hydroxyphenylacetic acid (m-OHPAA and p-OHPAA, respectively). BA and PAA are present in urine as their amino acid conjugates, hippuric acid and phenylacetylglutamine, respectively (130,131). Prior to the isolation of these two acids, these conjugates must be hydrolyzed. Several hydrolytic methods are available. Hippuric acid was readily hydrolyzed in either strong alkali (190) or strong acid (191) heated to 100°C for at least 1 h. Similarly phenylacetylglutamine was hydrolyzed, liberating PAA, in strong acid (6N HCl) heated to 100°C for 2 h (192). Typical solvents used for the extraction of these acids (PAA,BA) included ethyl acetate (193), chloroform (194) and dichloromethane (195). Ion exchange resins such as DEAE-Sephadex have also been used to isolate organic acids including hippuric acid, BA, and p-OHPAA from urine (196).

1.4.1.3 Isolation of Volatile Metabolites

EBZ has been proposed as a metabolite of PLZ, which may be excreted in the expired air. The volatile nature of this compound required the development of a method which could isolate this metabolite from samples of expired air. Several procedures for the isolation of volatile compounds from air samples have been published in the literature. The methods described utilize either solid adsorbants or cryogenic traps to isolate these compounds. Several types of solid adsorbants have been used (Porapak®, activated carbon, XAD and Tenax GC®) (197). Tenax GC® is the most frequently used adsorbant. One study utilized this adsorbant to isolate toluene from the expired air of people exposed to this solvent (198). Cryogenic traps using supercooled solvents have also been used for the isolation of volatile compounds from expired air. Radiolabelled propane was isolated from expired air of rats by bubbling the air sample through diethyl ether cooled in a liquid nitrogen bath (107). The trapping efficiency of this procedure was estimated to be 70%.

1.4.1.4 Isolation of Amphoteric Compounds

A number of the metabolites of LID are amphoteric, having both acidic and basic properties. Even with appropriate pH adjustment it is still difficult to extract these compounds efficiently from an aqueous solution, although this procedure is commonly used.

The compounds 3-OH-LID, 3-OH-MEGX and 4-OH-Xyl are observed in urine as glucuronide conjugates (115). Prior to the isolation of the hydroxylated metabolites of LID these conjugates must be hydrolyzed. Nation et al. (26) hydrolyzed the glucuronide conjugate of 4-OH-Xyl in 2 ml of urine by adding 1 ml of 10 M HCl and heating the resulting solution in a boiling water bath for 1 h. Hydrolysis of the conjugates of 4-OH-Xyl, 3-OH-LID and 3-OH-MEGX was accomplished by acidifying the aqueous sample with 3N HCl and heating at 100°C for a 15 min period (115). Enzymatic preparations containing β-glucuronidase and sulfatase have also been used to hydrolyze these conjugates (117). The procedure normally requires the enzyme-treated solutions to be incubated at 37°C for a 24 h period.

Following hydrolysis, the phenolic metabolites 3-OH-LID, 3-OH-MEGX and 4-OH-Xyl are generally isolated by solvent extraction. Nelson et al. (117) extracted 3-OH-LID and 3-OH-MEGX from urine samples after the aqueous solution was buffered to pH 8.5. Dichloromethane was used as the extracting solvent. 4-OH-Xyl could be extracted from a strong alkali solution (3N NaOH) into diethyl ether (26). Both Keenaghan and Boyes (115) and Adjepon-Yamoah (187) were able to extract 4-OH-Xyl from urine adjusted to pH 7.2. Dichloromethane was again used as the extracting solvent in both studies. It was found necessary to repeat this extraction procedure several times.

1.4.1.5 Isolation of Intact Glucuronide Conjugates

No procedure has been described for the isolation of the glucuronide conjugate of 4-OH-Xyl, a proposed metabolite of LID. Several methods, however, are available for the isolation of glucuronide conjugates of other compounds. The conjugate(s) of 4-OH-Xyl would be expected to behave in a manner similar to these other glucuronide conjugates. Previously used isolation methods include lead precipitation (199,200), direct solvent extraction (201-204), ion pair extraction (205), or the use of ion exchange resins (206-209). Adsorptive columns such as XAD-2 (206,208), silica gel (210) and Sep C-18® (211,212) have been utilized to isolate glucuronide conjugates from urine as has gel filtration using Sephadex LH-20 (207). Preparative high performance liquid chromatography (HPLC) is another convenient method for the isolation of glucuronide conjugates (201,203,206,213).

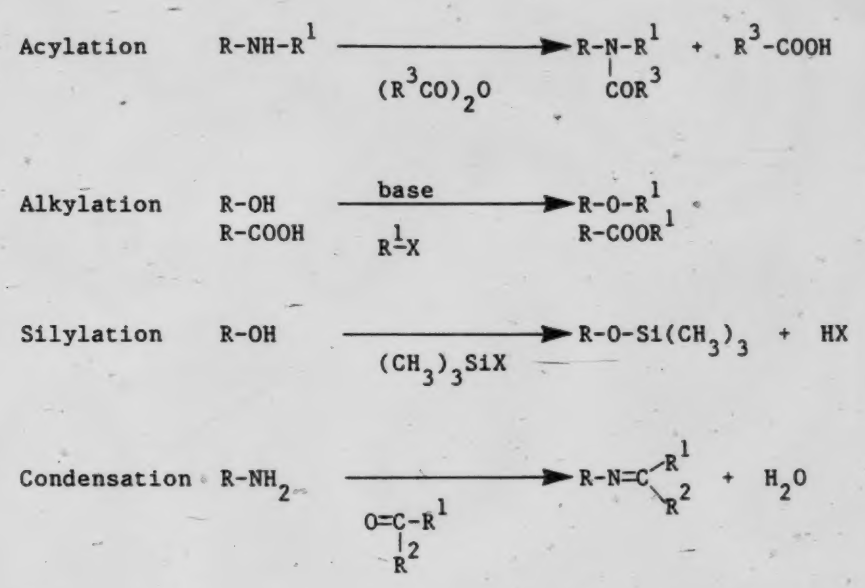
Thin layer chromatography (TLC) can be used to purify isolated glucuronide conjugates. Silica gel is the most popular stationary phase used in these procedures (204,208). Visualization of the separated glucuronide conjugates can be achieved using ultraviolet (U.V.) light (210,212) or naphthoresorcinol spray (212,214,215). This spray is specific for glucuronic acid containing compounds, which appear as blue spots when the sprayed TLC plates are heated.

1.4.2 Derivatization Methods

Compounds are derivatized for several reasons: a) to increase their extractability from aqueous solutions, b) to increase their volatility, c) to introduce specific groups which enhance detectability, d) to improve the stability of the compound or e) to improve chromatographic separation from other components present in the sample (216). Derivatization of alcohol, carboxylic or amino groups reduces the adsorption of these compounds by the GC column (217). The principle types of derivatization procedures used in GC are depicted in figure 4.

1.4.2.1 Derivatization of Bases

Acylation techniques have been applied to the derivatization of a variety of compounds, including PLZ and PEA. Reagents employed to acylate PLZ were trifluoroacetic anhydride (TFAA), heptafluorobutyric anhydride (HFBA) or acetic anhydride. Several products were formed when PLZ was reacted with TFAA, two of which were identified by mass spectrometry as N'-trifluoroacetylphenelzine and N,N'-ditrifluoroacetylphenelzine (175). Cooper et al. (174) derivatized PLZ with HFBA and determined that nanogram quantities of derivatized PLZ could be detected. PLZ was also converted under anhydrous conditions to its acetyl derivative using acetic anhydride (175). Two products were also formed with this reagent, identified as N'-acetylphenelzine and N,N'-diacetylphenelzine. Derivatization of PLZ for GC analysis has also been achieved by condensing PLZ with a variety of aldehydes and ketones. These procedures were carried out in aqueous solutions. In urine, PLZ was converted to the corresponding acetonide by reaction with acetone (162). The derivative formed was insensitive to an electron capture detector (ECD). Using a flame ionization detector (FID), 0.1 ug of PLZ/ml of sample was the maximum that could be detected by this procedure. To enhance the detectability of



Acylation $$R\text{-}NH\text{-}R^1 \xrightarrow[(R^3CO)_2O]{} R\text{-}\underset{\displaystyle COR^3}{\underset{|}{N}}\text{-}R^1 + R^3\text{-}COOH$$

Alkylation $$\begin{matrix} R\text{-}OH \\ R\text{-}COOH \end{matrix} \xrightarrow[R^1\text{-}X]{\text{base}} \begin{matrix} R\text{-}O\text{-}R^1 \\ R\text{-}COOR^1 \end{matrix}$$

Silylation $$R\text{-}OH \xrightarrow[(CH_3)_3SiX]{} R\text{-}O\text{-}Si(CH_3)_3 + HX$$

Condensation $$R\text{-}NH_2 \xrightarrow[\underset{\displaystyle R^2}{\underset{|}{O=C}}\text{-}R^1]{} R\text{-}N=C\begin{matrix} \diagup R^1 \\ \diagdown R^2 \end{matrix} + H_2O$$

Figure 4. Common types of derivatization reactions used in Gas Chromatography. Table reproduced from reference 218.

PLZ, fluorinated aldehyde and ketone reagents, pentafluorobenzaldehyde and benzoyltrifluoroacetone, were utilized (41,44). With either of these reagents, the optimal detection limit was 2 ng/ml of sample. Derivatives were detected using either GC/MS in the selective ion mode or GC with a nitrogen-phosphorus detector (NPD), respectively.

Acetylation of PEA in an aqueous solution, using acetic anhydride, is an efficient quantitative procedure which enhances the extractability of this compound from aqueous samples (219). The acetylated product is relatively insensitive to GC detection and has poor GC characteristics. To overcome these deficiencies, N-acetylphenylethylamine was further derivatized, under anhydrous conditions, using TFAA (219,16) or pentafluoropropionic anhydride (PFPA) (179). Both procedures yielded volatile derivatives which were extremely sensitive to ECD (detection limit 1 ng/ml). Pentafluorobenzoyl chloride (PFBC) (218,220,221) and pentafluorobenzenesulfonyl chloride (222) have also been used to derivatize PEA and other primary amines for GC analysis. Both reagents react with PEA in aqueous samples and yield derivatives which are sensitive to ECD at subnanogram levels. An aqueous dansylation procedure for the analysis of PEA by TLC and MS-direct inlet probe has also been described (223). Other reagents available for the aqueous derivatization of PEA include 2,4-dinitrophenylsulfonic acid, 2,6-dinitro-4-trimethylsulfonic acid and 2-nitro-4-trifluoromethylsulfonic acid (178). These two latter reagents were used to analyze PEA in the CNS tissue of rats with experimentally-induced phenylketonuria (224,177), but could not be used to measure normal levels of PEA in CNS tissue. PEA isolated from plasma, urine and cerebrospinal fluid has been analyzed as its isothiocyanate derivative, following its reaction with carbon disulfide (225). This derivative can be detected by either a NPD or FID.

1.4.2.2 Derivatization of Carboxylic Acid Metabolites

Esterification of organic acids can be achieved using several derivatizing reagents. These include diazomethane (226,227), 1,1'-carbonyldiimidazole and methanol (228), and N,N-dimethylformamide dialkylacetals (229). Several commercially available silylating reagents have also been used to derivatize organic acids. Both hippuric acid (230) and PAA (193,231,232) were analyzed by GC as their trimethylsilyl derivatives. The popularity of these silylating reagents for the derivatization of carboxylic acids is declining because the derivatives formed are susceptible to hydrolysis during the chromatographic procedure (230).

To enhance the detectability of organic acids, several halogenated derivatizing reagents have been utilized. Both m-OHPAA and p-OHPAA were derivatized with hexafluoroisopropanol. The phenolic group was further derivatized with PFPA (233). As little as 50 picograms of these acids could be detected using GC/MS in the selective ion monitoring (SIM) mode. Pentafluoropropanol has been used to derivatize PAA (234). PAA (235) and BA (236) could also be reacted with pentafluorobenzyl bromide. However, the limit of detection of PAA derivatized with this reagent was surprisingly high (20 ng on the column). Currently this reagent has only been used, in a quantitative study, to derivatize PAA isolated from plant material. Davis et al. (257) used a mixture of trifluoroethanol/PFPA to simultaneously derivatize carboxylic acid and phenolic groups. The resulting derivatives were detected, at low levels, using high resolution mass spectrometry (HRMS).

1.4.2.3 Derivatization of Lidocaine Metabolites

There are few procedures published in the literature describing the derivatization of the metabolites of LID. Those currently available are effective only when the derivatization reactions have been carried out under anhydrous conditions, which require prior extraction of the phenolic amine metabolites from the aqueous biological samples. Keenaghan and Boyes (115) extracted 4-OH-Xyl into dichloromethane prior to its conversion to a heptafluorobutyryl derivative with heptafluorobutyrylimidazole. Acetylation of 4-OH-Xyl, using acetic anhydride under anhydrous conditions, and 3- and 4-OH-LID, using the reagent N-acetylimidazole have been reported (238). The low sensitivities of the procedures in current use are attributed to the poor extractibility of these compounds and the poor selection of the derivatizing reagents. Low level analysis of these compounds has not been achieved.

1.4.2.4 Derivatization of Doxapram Metabolites

There is only one reported procedure where a metabolite of DOX, 1-ethyl-4-(2-ethylamino)-3,3-diphenyl-2-pyrrolidinone (1b; Fig. 3), was reacted with acetyl chloride under anhydrous conditions. The product formed was then separated, from other components present in the sample, by TLC (91).

1.4.2.5 Derivatization of Glucuronide Conjugates

Derivatization of isolated glucuronide conjugates is necessary to enhance the volatility of these compounds for GC and GC/MS analysis. A procedure used successfully involves the acetylation of the glucuronide conjugates under anhydrous conditions after the carboxylic acid group has been converted to its methyl ester using diazomethane (127). Glucuronide conjugates have also been converted to their trifluoroacetyl derivatives, using N-methyl-bis(trifluoroacetamide) (239) to increase their volatility. Trimethylsilylating reagents are frequently used to derivatize glucuronides (203,206,209,210). These reagents readily react with both alcoholic and carboxylic acid groups, producing derivatives of significantly greater molecular weight. These trimethylsilylated glucuronide conjugates require high temperatures for their elution from GC columns during the chromatographic procedure. Partial hydrolysis of acyl and ether glucuronides can occur during the derivatization procedure (127). Permethylation with methyl iodide in a strong base (e.g. sulfinylmethide carbanion) is another procedure which was used for the derivatization of glucuronide conjugates (240). Both alcohol and carboxylic acid groups are derivatized by this procedure. Partial hydrolysis of ester glucuronides and amide bonds has been observed with this reagent (127,240). In addition, amine groups may react with this reagent forming a quaternary salt (127). This latter problem can be overcome by initially converting the primary or secondary nitrogen to its respective amide (241).

1.4.3 Gas Chromatography

GC is a convenient method for the separation and detection of a mixture of volatile compounds. Since the GC separation procedure is generally carried out at an elevated temperature, compounds analyzed by this technique must be thermally stable.

GC analysis was utilized for the separation of DOX and its metabolites (28,40,45). To optimize separation of these compounds, appropriate column selection was critical. Using an OV-17 column, it was found that DOX was poorly resolved from its metabolites 11 and 1-ethyl-4-(2-ethylamino)-3,3-diphenyl-2-pyrrolidinone (1b; Fig. 3) (45). Although improved separation was achieved using an OV-225 stationary phase the compounds still had poor chromatographic characteristics.

GC separation of derivatized glucuronide conjugates was achieved using several different stationary phases. These include, 1-10% OV-1 (133,206), 1-10% OV-101 (198) and SE-30 (242). High column temperatures were required for the elution of these derivatized conjugates.

The choice of GC detector depends on the properties of the compound itself and of the derivatives which have been prepared. ECD, FID and NPD have all been used for the analysis of PLZ and PEA isolated from biological samples depending on the property ~~of the derivatives~~ that were formed. ECD are used infrequently to analyze PAA, even though several of the reagents used to derivatize this acid contain halogen atoms. Those derivatives formed with silylating or methylating reagents are generally detected with a FID (177). To date no one has used ECD-sensitive reagents for the derivatization of the metabolites of LID. Routine analysis of these compounds are by a FID (115) or MS (117). DOX and several of its metabolites have been detected, following GC separation, using a NPD (40).

1.4.4 Mass Spectrometry

MS is used in drug metabolism studies for both detection and identification of compounds, following their separation by GC. Compounds are identified by the presence of diagnostic fragment ions. Normally, confirmation of the identity of a compound is achieved by comparing its fragmentation pattern with that of an authentic standard.

Coutts et al. (219) investigated the mass spectral characteristics of perfluoroacyl derivatives of some arylalkylamines, including N-acetyl-N-trifluoroacetylphenylethylamine. Similarly, metabolites of DOX, isolated from the urine of dogs, were identified by their electron impact (EI) fragmentation pattern (121). Derivatization of glucuronide conjugates and their subsequent separation by GC allowed the identification of these compounds by MS. Several derivatized glucuronide conjugates have been identified in this manner (203,206,212,240,242). Underivatized clofibric acid glucuronide was identified by MS using the direct inlet probe technique (215). Permethylated glucuronide conjugates are readily identified by electron-impact mass spectrometry (EI/MS) since the glucuronide moiety yields a number of diagnostic fragment ions (127). Chemical ionization-mass spectrometry (CI/MS) has also been used to identify glucuronide conjugates of several compounds. The conjugates of lorazepam and oxazepam both gave prominent quasimolecular ions using CI/MS (243).

Underivatized glucuronide and sulfate conjugates could be identified using fast atom bombardment-MS (129,208,209,212,244). This technique was capable of identifying ammonium-linked glucuronide conjugates (208).

Confirmation of the origin of certain metabolites in metabolism studies, was achieved using MS. Dyck et al. (98) confirmed that PEA and PAA, two compounds normally formed during intermediary metabolism, were also *in vivo* metabolites of PLZ. This was achieved by administering d_4-PLZ. The presence of the deuterium atoms in PEA and PAA was confirmed by mass changes in certain fragment ions obtained during EI fragmentation of these compounds.

Mass fragmentography is a technique, used in metabolism studies, where the mass spectrometer is used as a sensitive detector. In this technique selected fragmentation ions are monitored (SIM). Deuterated analogs of the compound being analyzed are usually used as an internal standard. PLZ was quantitated in plasma derivatized with acetic anhydride (175) or with pentafluorobenzaldehyde (41) using SIM. Similarly PEA, as its dansyl derivative, could be quantitated in brain tissue at very low levels (1 ng/g) using mass fragmentography (223). PAA in human plasma (204) and m-OHPAA and p-OHPAA in rat brain (211) were all analyzed by HRMS-SIM. CI/MS-SIM has been used to quantify the metabolites of LID in human urine. The internal standard used was labelled with deuterium atoms. All the compounds analyzed were introduced into the mass spectrometer by direct-inlet probe (118). Quantitation of DOX in the plasma of premature infants was achieved using CI/MS-SIM by monitoring for the quasi-molecular ion, m/z 379 (45). The technique was capable of detecting 10 ng of material in 500 ul of plasma.

1.4.5 High Performance Liquid Chromatography (HPLC)

The use of HPLC for the separation and quantitation of acidic compounds has increased in popularity since prior extraction of these compounds is not required. Hippuric acid (245) and PAA (246) have been analyzed by HPLC, using U.V. detection. The limit of detection of PAA in urine was 28 ng/ml.

The separation and quantitation of conjugated xenobiotics was also achieved using HPLC. Aqueous samples are analyzed by this technique, so tedious clean-up, extraction and purification procedures are not required. The use of Sep-pak® octadecylsilyl columns and subsequent elution with methanol (211) or phosphate buffer/acetonitrile (212) are often used for sample clean-up prior to injection into the HPLC.

1.4.6 Other Methods of Analysis

Thin layer chromatography (TLC) was used successfully to separate PEA from other endogenous constituents isolated from brain tissue. Following the separation of PEA, as its dansyl derivative, on silica gel TLC plates, the compound was isolated and quantitated by MS using a direct inlet probe (223). Spectrophotometry, at 254 nm, could also be used to quantitate PEA following its separation from other endogenous components present in the extract by TLC

(247).

Organic acids could also be separated using TLC plates. Hippuric acid was separated from other urinary components on silica gel. This metabolite was visualized using 5% p-dimethylaminobenzaldehyde. Quantitation was achieved, using a densitometer, by comparison with standard quantities of hippuric acid (248). BA, PAA and several other organic acids were separated by TLC following their conversion to anilides. Either alumina or polyamide TLC plates could be used for the separation (249).

TLC plates of inactivated silica gel were used to separate DOX and its metabolites. Methanol or ethanol proved to be satisfactory mobile phases. Visualization of the separated metabolites was accomplished by spraying the plates with aqueous potassium permanganate or by the fluorescence produced when the plates were irradiated with short wavelength U.V. light (91).

TLC was also used for the separation and isolation of conjugated compounds. Conjugates must first be solubilized in a volatile solvent for application of these compounds to the TLC plate. Jannsen et al. (209) separated the ammonium-linked and acyl glucuronide of 3-(p-chlorophenyl)-thiazolo (3,2a)-benzimidazole-2-acetic acid on silica gel. Visualization of these conjugates on the TLC plate is usually with U.V. light or with naphthoresorcinol spray (203,208,212).

DOX was analyzed by U.V. light absorption following its oxidation to benzophenone. The oxidation was carried out with potsssium permanganate (91). However, other metabolites derived from DOX interfered with the assay. DOX itself cannot be quantified by U.V. light absorption since its absorbance is too low to permit its analysis in biological samples. The absorption maxima of DOX were observed at 269, 264.5, and 253 nm. Other DOX-related materials which have an intact diphenylpyrrolidinone moiety gave the same U.V. spectrum and therefore would also interfere with the assay (91).

A radioenzymatic assay was developed for the analysis of PEA in brain tissue. The technique involved isolating PEA by extraction and then converting it to phenylethanolamine with the enzyme dopamine-β-hydroxylase. This product is subsequently methylated using the enzyme phenylethanolamine N-methyl transferase. The methyl donor used was ^{3}H-S-adenosyl-methionine, labelled on the transferable methyl group (250). The quantity of PEA present was proportional to the amount of radioactivity associated with N-methyl-phenylethanolamine. This technique permitted the quantitation of PEA in control brain tissue (1 ng/g).

Caddy et al. (42) measured PLZ in biological fluids after its oxidation to 2-phenylethanol with potassium iodate. The major disadvantage of this technique was that several other metabolites interfered with the assay. Those metabolites which retain the hydrazine moiety, for example, are also converted to 2-phenylethanol by this procedure.

1.5 *In Vivo* and *In Vitro* Animal Models of Drug Metabolism

1.5.1 *In Vitro* Models

1.5.1.1 Isolated Rat Liver Perfusion

Isolated rat liver perfusion experiments offer a convenient means of studying the liver's contribution to the metabolism of a drug. Since both organ and cellular integrity are maintained, these studies more adequately represent the *in vivo* situation than do studies with microsomes or isolated cell preparations. In addition, this preparation allows the infusion of ~~drugs at~~ higher concentrations than are normally administered *in vivo* (251).

A number of metabolism studies have been initiated using this organ preparation (252-254). Metabolic reactions which have been investigated using the isolated rat liver preparation include N-demethylation (251), O-demethylation (251), reduction (255), hydroxylation (256) and conjugation (257). Since competing metabolic reactions can still occur with these preparations, compounds may undergo a variety of biotransformations. For example, eight different metabolites were detected when 2-(n-propyl)-4-pentenoic acid was infused into an isolated rat liver (254).

The portal vein or the hepatic artery, lower inferior vena cava and upper inferior vena cava are surgically cannulated with polyethylene tubing . Following cannulation, the liver is either removed from the rat (258) and placed in a perfusion chamber or the liver perfusion is performed *in situ* (252). The perfusion buffer and drug are infused through either the portal vein or hepatic artery cannula. Eluate is collected from the upper inferior vena cava. Oxygen consumption is monitored through the lower inferior vena cava.

The liver requires an adequate perfusion flow rate to maintain liver viability and ensure proper tissue oxygenation. The perfusion flow rate depends on the physical composition of the perfusion medium. Low flow rates (10 ml/min) (257) can be used provided the perfusion media contains washed erythrocytes which improves oxygenation of the liver. Higher flow rates (20-30 ml/min) are required if the erythrocytes are not present (255,258). Commonly used perfusion buffers include Kreb's-Ringer (255), Kreb's-Heinseleit (252) and Kreb's-bicarbonate (257) buffer. Bovine serum albumin and glucose are often added to the perfusion media to provide proper osmolality (253,255,257). When the livers are perfused through the portal vein, flow can be generated by gravity driven hydrostatic pressure. A Harvard apparatus variable speed peristaltic pump is used to establish flow when infusion is through the hepatic artery (259).

Perfusate may be passed through the liver once only or it may be recycled (258). Although the recycling of perfusate will more truly reflect drug elimination after an i.v. bolus dose, this technique has certain drawbacks. The perfusate used with this technique must contain red blood cells for proper aeration of the organ. With this method metabolites and other endogenous by-products will slowly accumulate in the perfusate during the experiment. As these products accumulate, certain metabolic pathways may be affected by product inhibition. In both systems, oxygen is supplied by aerating the perfusate with an O_2/CO_2 (95:5) mixture. The pH is also maintained at 7.4 using an automatic pH titration unit.

The most important consideration with regard to the utility of the isolated organ preparation in metabolism studies is the liver's continued viability following isolation and prolonged infusion. A variety of tests have been devised to establish the functional condition of the liver. The most drastic method is to reconnect the perfused liver into the donor animal and determine its functional capacity (260). However, investigators have developed a number of alternative tests to monitor viability. Appearance of the liver is an important consideration. Livers should not appear swollen and the edges should remain thin with no obvious black spots appearing during perfusion (260,261). Levels of the enzymes pyruvate-glutamate transaminase and glutamate-oxalate transaminase in the effluent or the activity of acid phosphatase have been used to monitor liver viability (262). The presence of lactate dehydrogenase in the perfusate indicates liver damage (263). Other methods used to evaluate liver function include glucose production, potassium efflux, and the ratio of lactic acid to pyruvic acid (252,257,264). The simplest methods used to monitor liver function are oxygen consumption, stable production of metabolites and a stable perfusion flow rate (261,263,265).

Isolated rat liver perfusion preparations should not be used to investigate biliary excretion, since bile flow decreases significantly during perfusion (252).

Livers have been maintained in a viable state for periods of up to 5 h following removal of the organ from the animal (260,264,266). These preparations appear normal histologically even after 4 h (257). Bartosek et al. (262) demonstrated, however, that transaminase levels increased after 3 h of perfusion, which indicates a reduction in the viability of the liver.

The use of a non-recycling perfusion system allows drug metabolism to be investigated at steady state. The ability to measure the rate of drug metabolism at a constant rate of drug input is a unique advantage of this system over other preparations (i.e. cell preparations or microsomal preparations).

1.5.2 *In Vivo* Animal Models

1.5.2.1 Jugular Vein Cannulation

Drug metabolism and pharmacokinetic studies usually require the withdrawal of single or serial blood samples. Frequent blood sampling over prolonged periods of time is necessary to accurately evaluate the time course of a drug in the body. Several sampling techniques have been developed to enable frequent blood sampling in the rat.

Tail vein laceration or amputation of a portion of the tail is the simplest method available to obtain blood samples from the rat (267). This technique, however, is both painful to the animal and inconvenient for frequent sampling of blood. Rupturing the occipital vein, behind the orbital sinus, is another method used for serial blood sampling. Although more blood samples can be obtained by this method (compared to tail amputation) only small blood volumes may be withdrawn (267).

Cannulation of the jugular vein is a more complicated but well suited procedure for frequent blood sample collection from rats (267,268,269). Volumes of up to 1 ml per collection have been obtained from a jugular vein cannulated rat (268). The method also allows saline replacement of the blood volume removed from the animal during the collection period. During sampling, the animals move freely with no visible signs of stress. Rats have been maintained for up to 5 weeks with a surgically implanted jugular vein cannula. To prevent clotting, the cannula is filled with heparinized saline (10 units/ml) (268). Jugular vein cannulation offers a convenient method to study drug kinetics following single doses as well as after chronic administration of the drug.

1.5.2.2 Bile Duct Cannulation

Bile duct cannulation has been used to investigate the biliary excretion and enterohepatic recirculation of a variety of drugs and metabolites. The rate of drug elimination by this route is important because it is influenced by the presence of other drugs (270,271,272), by age of the recipient (273), by a disease state (274) or by the drug dose (275). Changes in biliary clearance may influence the blood level of a drug (276). Evaluation of these physiological processes requires a method which permits continuous bile collection.

The bile duct is normally cannulated approximately 1-2 cm from the hilus of the liver (277). The cannula is secured to the abdominal wall and then exteriorized through one of the following: the abdominal wall (278), the midline incision (279), dorsal cervical area (280), or the rear leg (281). All surgical procedures are conducted while the animal is anesthetized.

A variety of techniques have been devised to allow collection of bile from these animals. Currently, the most common methods are to maintain the animal under anesthesia throughout the duration of the experiment or to place the animal in a restraining cage following recovery from the anesthetic. Ether (282) and pentobarbital (281,283-287) have been used to maintain rats under anesthesia during the experiment. These agents have been found to reduce the extent of drug conjugation (288) and bile flow (75). A consequence of ether anesthesia is that the concentration of UDPGA in the liver is reduced to 70-90% of control levels. A reduction of UDPGA is also observed following administration of pentobarbital, urethane, halothane, enflurane or methoxyflurane. In addition to the effect of the anesthetic agent, these animals may be traumatized to the extent that the bile flow is altered. Artificial ventilation, required to maintain these animals, is provided through a tracheotomy (283,284) and body temperatures are maintained with incandescent lamps (283,287). Dehydration is prevented by slowly infusing these animals with a Kreb's-bicarbonate buffer (277). The maximum duration of experiments using this animal model is 2-3 h since prolonged anesthesia reduces the blood pressure of the animal. Reduction in blood pressure will cause a reduction in bile flow (277) and any alteration in bile flow will invariably influence the rate a drug is excreted by this route.

To minimize the undesirable effects of surgery and anesthesia on biliary excretion, several studies were conducted using rats which had recovered from surgery and were maintained in Bollman restraint cages (280, 289-292). This cage restricts the movement of the animal and prevents the animal access to the cannula. To conserve bile salts following surgery a re-entry cannula is inserted into the duodenum which allows normal recycling of the bile (272). During experiments, the cannulae are disconnected and bile collected. Although some investigators have maintained rats in these restraint cages for several weeks (289), this procedure is not practical. Typically, this method is used for single dose drug studies.

Few methods have been developed which allow collection of bile from fully recovered, freely moving animals. Vonk et al. (293) developed a technique by which the bile duct was cannulated and the cannula exteriorized behind the head. This cannulae was attached to a 55 cm length of narrow bore polyethylene tubing. Where these cannula are connected, a swivel joint was inserted which allowed the animal free movement within the cage. The cannula was attached to the back of the skull using dental cement. Animals cannulated in this manner were maintained for several months (293). This technique, however, does not permit recycling of bile. A modification using a double swivel joint has been developed which allows simultaneous infusion of bile salts and collection of bile in a manner similar to the method just described (288). However, recycling of bile using this method has never been demonstrated. This method does have practical limitations; the excessive length of tubing increases the risk of bile salt precipitation (which can block the bile flow) and a higher pressure is required to generate bile flow.

Another technique involves the implantation of a 12 ml glass vessel into the peritoneal cavity (278). The bile duct is cannulated and the cannula inserted into this glass vessel, so that bile can be collected. The vessel has a side arm, exteriorized through the abdominal wall, from which samples can be withdrawn. These bile duct-cannulated animals can be housed in commercially available metabolic cages so that simultaneous collection of bile, urine and expired air is possible. Dehydration and loss of bile salts are potential problems with this technique, as with the other techniques just described.

Bile flow rates, although quite variable between different studies have been estimated to be 0.8-1.0 ml/h in rats (277,294). With prolonged collection of bile, the net loss of bile salts will cause a reduction in bile flow. Decreases in bile flow can affect the rate at which a compound is cleared in bile (287). To prevent this decrease in bile flow some investigators have infused sodium taurocholate (4 mM) at a rate of 12 ml/h/kg, to replenish the lost bile salts. The rate at which this compound is infused is critical since higher infusion rates may cause choleresis, doubling the bile flow rate (277). Sodium chloride solution (0.9%, 4.5 ml/h/100 g) has been used to replace fluid lost when bile is collected for long periods (295). Simple replacement of the lost fluid, however, does not prevent the reduction in bile flow observed during prolonged collection.

1.6 Rational

A research area which has expanded in recent years is the metabolism of drugs. Drug metabolism is now the subject of extensive reviews (93,297). Principally these studies were required to fully evaluate the toxicological and pharmacological effect of drugs. Often metabolites are formed which are pharmacologically active. It is hoped that with additional knowledge on drug metabolism, newer and and safer therapeutic agents may be developed.

Metabolism also plays a significant role in drug elimination. Often with drugs which are extensively metabolized in the body, dose- and time-dependant changes in eleimination can occur. Often associated with this change in elimination is an alteration in the pattern the drug is metabolized. If this results in the increased production of toxic metabolites, serious side effects may be observed during drug therapy.

Essential in the study of drug metabolism is the development of appropiate analytical techniques for the isolation and identification of metabolites. Knowledge in the area of drug metabolism often parallels new technologies in analytical chemistry. In this study TLC, GC and GC/MS were all utilized to determine the metabolism and, where appropriate, the kinetics of three drugs PLZ, LID and DOX. These drugs were selected for study on the basis of 3 criteria a) current lack of knowledge regarding their metabolism, b) the need to develop an appropriate analytical technique for the analysis of these drugs and/or their metabolites in biological samples and c) the importance metabolism appears to play in the elimination of these drugs.

2. Material and Methods

2.1 Reagents

2.1.1 Commercial Sources

Ethyl acetate, toluene, sodium citrate, 1-naphthoic acid, m-aminobenzoic acid, o-methoxybenzoic acid and sodium hydride were obtained from British Drug Houses (Poole, England). Pentane, potassium bicarbonate, potassium carbonate, magnesium sulfate, dimethyl sulfoxide, benzoic acid and phenylacetic acid were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Pierce Chemicals (Rockford, IL., U.S.A.) was the source of trifluoroacetic anhydride, trifluoroacetic acid, pentafluoropropionic anhydride, heptafluorobutyric anhydride and hexamethyldisilazane. Ethyl chloroformate, phenelzine sulfate, 2-phenylethylamine hydrochloride, xylidine, benzylhydrazine hydrochloride, ethylbenzene, decadeuterated ethylbenzene, ethanolamine, 2-methoxyflurane, methylethylketone, pentafluorobenzoyl chloride and pentafluoro-benzenesulfonyl chloride were products of Aldrich Chemical Company (Milwaukee, WI., U.S.A.). Di-(2-ethylhexyl)-phosphoric acid, p-hydroxyphenylacetic acid and m-hydroxyphenylacetic acid were products of Sigma Chemical Company (St. Louis, MO., U.S.A.). Terochem (Edmonton, Alta., Can.) was the source of cyclohexane and acetic anhydride. Dichloromethane was obtained from General Intermediates (Edmonton, Alta., Can.). Eastman-Kodak Company (Rochester, N.Y., U.S.A.) was the source of D,L-phenylalanine and m-methoxybenzoic acid. Aquasol-2, ^{14}C-phenylethylamine and ^{14}C-5-hydroxytryptamine were purchased from New England Nuclear (Boston, MA., U.S.A.). Doxapram hydrochloride and diazepam hydrochloride were obtained from A.H. Robins (Mississauga, Ont.,Canada.) and Hoffmann-LaRoche (Vaudevil, P.Q., Can.), respectively. 1-^{14}C-Phenylacetic acid was obtained from Amersham (Oakville, Ont., Can.). Travesol® was obtained from Baxter Travenol Laboratories (Malton, Ont., Can.).

2.1.2 Synthesized Standards and Gifts

4-Hydroxylidocaine, 4-hydroxymonoethylglycinexylidide and 4-hydroxyglycinexylidide were prepared by Drs. R.T. Coutts and Loc Van Chu. 1-^{14}C-Phenelzine sulfate was prepared from 1-^{14}C-phenylacetic acid by Dr. T.W. Hall. 1-Phenylcyclopentanoic acid was a gift from the Defense Research Board, Ottawa. Astra Pharmaceuticals Ltd. (Mississauga, Ont., Can.) kindly provided lidocaine and its metabolites monoethylglycinexylidide, glycinexylidide, 3-hydroxylidocaine, 3-hydroxymonoethylglycinexylidide and 4-hydroxy-2,6-xylidine.

2.1.3 Distilled Water

Deionized, distilled water was prepared by passing glass distilled water through a series of ion exchange and adsorption resins in a Millipore Milli-Q® reagent water system (Bedford, MA., U.S.A.).

2.2 Apparatus

2.2.1 Gas Chromatography

Capillary GC analysis was performed using a Hewlett-Packard model 5730A gas chromatograph (Palo Alto, CA., U.S.A.) or a Varian Vista 6000 GC (Georgetown, Ont., Can.). Several instruments were used each equipped with a different detector: a 15 mCi ^{63}Ni source for electron capture detection; a NPD; or a FID. All the gas chromatographs were equipped with a HP-18740b capillary column inlet control system. GC peak areas were integrated using a HP-3390A integrator.

2.2.2 Mass Spectrometry

Mass spectra of compounds were recorded using a Hewlett-Packard model 5980A mass spectrometer linked to a 5934A 21 MX computer for data processing. This system permitted only electron impact mass spectra to be generated. In addition, both electron impact and chemical ionization spectra were recorded [1] on a VG micromass 7070E mass spectrometer linked to a Varian Vista 6000 GC and a PDP 11 data system (Analytech Instrumentation and Service, St. Laurent, Can.).

2.2.3 Other Instruments

TLC separation was achieved on Kieselgel 60 TLC plates (Camag, Hamburgerstrasse, Switzerland).

During solvent extraction of aqueous samples the immiscible phases were mixed in either a Labquake® (Labindustries, Berkeley, CA., U.S.A.) or in a multitube vortex mixer (IKA-Vibrax-VXR, Terochem, Edmonton, Alta., Can.).

Centrifugation of samples was performed on a floor model B20-A centrifuge (International Equipment Company, Needham Heights, MA., U.S.A.), a Clay-Adams bench top clinical centrifuge (Becton, Dickinson and Co., Parsippany, N.J., U.S.A.) or a Microfuge B (Beckman Instruments, Palo Alto, CA., U.S.A.).

Evaporation of the solvent, to concentrate the extract, was achieved using a Savant Speed Vac Concentrator/Evaporator (Emerston Instruments, Scarborough, Ont., Can.).

Preparation of tissue homogenates for measurement of MAO activity was done using a teflon tissue homogenizer, Fisher Scientific (Fairlawn, N.J., U.S.A.).

A number of instruments were used in the isolated rat liver perfusion studies to monitor liver function or to infuse the drug at a constant rate. Drug was infused at a preset rate using a model 600 Harvard Infusion/Withdrawl Pump (Harvard Apparatus, Southwick, MA., U.S.A.). During perfusion studies, the pH of the perfusion buffer was maintained at 7.4 using a Radiometer model TTT80 automatic titration system (Laboratory Equipment Div., London, Ont., Can.). The oxygen consumption of the liver was monitored using a Oxygen monitor YSI model 53 (Yellowsprings Instrument Co., Yellowsprings, OH., U.S.A.).

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[1] Recording of the mass spectra on the VG 7070E mass spectrometer was done by Dr. Alan Hogg and his research group, Department of Chemistry, University of Alberta.

For radioactivity (^{14}C) measurements a Beckman LS 7500 microprocessor controlled liquid scintillation counter (Palo Alto, CA., U.S.A.) was used.

2.2.4 Surgical Equipment

Sutures used to close incisions following the cannulation procedures consisted of blue polyester 0 Ti-cron® thread (American Cyanamid Co., Pearl River, N.Y., U.S.A.). The Ethicon® 3-0 silk thread used for stay sutures and to tie the jugular and bile duct cannulae in place was purchased from Ethicon Sutures Ltd. (Peterborough, Ont., Can.). A number of different cannulae were implanted into animals for collection of various biological fluids.

1. PE-50 was used for the cannulation of the portal vein in the isolated perfusion study and the jugular vein for *in vivo* experiments.
2. PE-10 intramedic tubing was used for the cannulation of the lower inferior vena cava for withdrawal of effluent, during the isolated liver perfusion study, to measure oxygen consumption.
3. PE-10 Tygon® tubing (0.01 X 0.03 in) was used for the cannulation of the bile duct of rats.
4. The cannulation of the upper inferior vena cava, to collect effluent from isolated perfused rat liver, was achieved using PE-100 intramedic tubing.

The intramedic tubing was purchased from Dynac (Becton Dickinson and Co., Parsippany, N.J., U.S.A.), while the PE-10 Tygon® tubing used for the cannulation of the bile duct was obtained from Fisher Scientific (Fairlawn, N.J., U.S.A.). The actual cannula used for the jugular vein cannulations consisted of a length (30 cm) of PE-50 tubing joined to a 33 mm piece of Silastic® tubing. The joint consisted of inserting 4 mm of the PE-50 tubing into the Silastic® tubing. Only the Silastic® tube was inserted into the jugular vein. The Silastic® tubing was a product of Dow Corning (Midland, MI., U.S.A.). For the collection of urine, feces and expired air from rats, the animals were housed in glass metabolism cages (Stanford Glass Co., Palo Alto, CA., U.S.A.).

2.3 Gas Chromatographic Procedures

Several GC columns and conditions were used for the separation and detection of the compounds of interest. The methods used are outlined in table 1. All columns were purchased either from J & W Scientific (Rancho Cordova, CA., U.S.A.) (Durabond columns) or from Hewlett-Packard (Palo Alto, CA., U.S.A.) (OV columns).

Helium (1 ml/min, set at the highest oven temperature used) was used as carrier gas for all GC and GC/MS procedures. The other gases and their corresponding flow rates are as follows: premixed argon/methane (95:5) (30 ml/min) was used as a makeup gas for EC detection, the combustion of a mixture of hydrogen (3 ml/min) and air (50 ml/min) was used to form the flame used for NP detection, while combustion of a hydrogen and air mixture was used to establish the ionizing flame for flame ionization detection. The injection port temperature was set at 250°C for all the GC procedures, while the detector temperature was set at 250°C for the ECD and 300°C for the FID and NPD.

2.4 Glassware Cleaning

All glassware was thoroughly cleaned prior to use. The method used involved scrubbing the glassware with a plastic bristle brush. Once done, the glassware was then rinsed repeatedly with distilled water followed by methanol and finally dichloromethane. Following the solvent rinse, the glassware was allowed to air dry and then was heated in a muffle furnace, at 300°C, for at least 1 h.

Glass syringes used to inject samples into the GC were periodically cleaned in 3N HCl followed by successive rinses with methanol and ethyl acetate.

The GC injection port glass liners were regularly cleaned by soaking overnight in concentrated HCl, followed by thorough rinsing with water and then methanol. They were dried in an oven at a temperature not exceeding 150°C. Silylation of these liners was not done routinely. However, when necessary the liners were heated to 150°C for 4 h in a sealed tube containing 100-200 ul of hexamethyldisilazane.

Table 1. The GC conditions used for the analysis of derivatized phenelzine, lidocaine and their respective metabolites. Doxapram and its metabolite were analyzed in an underivatized form.

Compound	Column	Temp range (^{o}C)	Initial Temp Hold (min)	Temp Program Rate (^{o}C)	Detector
Phenelzine	OV-101 20m DB-1 30m	110-250 "	4	8	ECD
2-Phenyl-ethylamine	Ov-101 20m	80-250	2	16	ECD
Phenylacetic Acid	OV-101 20m	80-250	2	16	ECD
Doxapram	DB-5 9m	150-250	0	16	NPD
Lidocaine metabolites					
Pentafluoro-	DB-1 30m	180-280	2	8	ECD
benzoylated	DB-1701 30m	80-270	2	6	ECD
Acetylated	DB-17 20m	100-260	2	16	FID
Glucuronide of 4-hydroxy-2,6-Xyl	DB-17 10m	200-250	2	8	MS
Ethylbenzene	OV-101 10m	50-150	2	4	MS

2.5 Solvent Distillation

All solvents were glass distilled and stored in glass round bottom flasks. Solvents were routinely redistilled on a weekly basis. The distillation of trifluoroacetic acid was done over phosphorus pentoxide under a stream of nitrogen. Distillation of dimethyl sulfoxide was performed under reduced pressure.

2.6 Phenelzine Experimental

2.6.1 Isolation of Phenelzine and Phenylethylamine

Blood samples (10-500 ul) were diluted to 2 ml with distilled water, to which was added the internal standard (100 ul of a 4 mg/100 ml aqueous solution of benzylhydrazine hydrochloride for PLZ determinations or 20 ul of a 1.6 mg/100 ml aqueous solution of p-chlorophenylethylamine hydrochloride for PEA assays) and enough concentrated perchloric acid to make a 0.4M solution. The precipitated protein was removed by centrifuging the sample at 10,000 rpm for 15 min at 4°C, in a B-20A centrifuge. The resulting supernatant was transferred to a clean small culture tube (13 mm X 150 mm) and extracted with 4 ml of ethyl acetate. The organic layer was discarded and the supernatant basified to pH 8.0 with potassium bicarbonate. The resulting potassium perchlorate precipitate was removed by again centrifuging the sample at 3500 rpm for 10 min. The supernatant was transferred to a large clean culture tube (15 mm X 150 mm), 400 ul of a 0.025 M sodium phosphate solution (pH 7.8) was added and the basic sample extracted with 5 ml of chloroform containing the ion pairing reagent di-(2-ethylhexyl)phosphoric acid (2.5% v/v). The phases were mixed, for 10 min, using a Labquake® mixer. This was followed by a 10 min centrifugation in a Dynac bench top centrifuge. The aqueous layer was discarded and 0.5N HCl (2.5 ml) was added to the remaining organic layer. The phases were again mixed in the Labquake® mixer and then centrifuged for 10 min in a Dynac bench top centrifuge (Speed setting 100). The top aqueous phase was retained, and transferred to a clean large culture tube. This solution was then basified to pH 8.0 with potassium bicarbonate and the amines present derivatized by the addition of 300 ul acetic anhydride. Once the effervescence had stopped the derivatives were extracted into 4 ml ethyl acetate. This extract was retained until the samples were derivatized according to the procedure described in section 2.6.6.

2.6.2 Isolation of Phenelzine from Bile

Bile samples were collected from male Sprague-Dawley rats and frozen at -26°C until analyzed. After the samples were thawed, 10-20 ul of the collected bile was transferred to a clean small culture tube (13 mm X 100 mm). This sample was made up to 0.5 ml with deionized water to which was added 0.5 ml concentrated HCl and 100 ul of the internal standard (benzylhydrazine) solution, prepared as previously described (Section 2.6.1). The solution was incubated for 1 h at 100°C. After the hydrolysis procedure, 1 ml of a sodium hydroxide solution (7.5 g/100 ml) was added to the tube followed by 4 ml of ethyl acetate. The immiscible phases were mixed for 5 min in a Labquake® mixer and then centrifuged for 5 min. The organic layer was discarded. The remainder of the procedure was as described for the isolation of PLZ from blood (Section 2.6.1) starting with the addition of potassium bicarbonate, followed by centrifugation to remove the insoluble potassium perchlorate salt.

2.6.3 Isolation of Phenelzine from Perfusate

To the effluent (2 ml) obtained from a rat liver infused with a solution of PLZ dissolved in Kreb's bicarbonate buffer was added 50 ul of the internal standard solution (4.5 mg p-chlorophenylethylamine hydrochlride/100 ml deionized water), enough potassium bicarbonate to make a saturated solution and 300 ul of ethyl chloroformate. The solution was mixed in a multi-tube vortex mixer for 30 min. After this time period 4 ml of ethyl acetate was added to the tube. The immiscible phases were mixed for 10 min in the multi-tube vortex mixer and then centrifuged for 10 min in a Dynac bench top centrifuge (Speed setting 100). Following centrifugation the organic layer was transferred to a clean small culture tube and evaporated to dryness in a Savant Speed Vac Concentrator/Evaporator. The dried residue was reconstituted with 50 ul of ethyl acetate. One microliter of this extract was injected onto the GC linked to a NPD. The conditions used to separate derivatized PLZ, PEA and p-chlorophenylethylamine are described in table 1. Standard curves of PLZ and PEA were obtained by analyzing known quantities of PLZ or PEA dissolved in Kreb's bicarbonate buffer, by the procedure just described. The actual calibration graphs were produced by plotting the ratio, obtained by dividing the integrated peak area of PLZ by that of the internal standard, versus the corresponding concentration of PLZ. The standard curve of PEA was obtained in the same manner.

2.6.4 Isolation of Phenylacetic Acid

Phenylacetic acid, its m- and p-hydroxylated analogs and benzoic acid were readily isolated from urine. The urine sample (50 ul) was diluted to 1 ml with deionized water, to which was added 1 ml of concentrated HCl and 30 ul of the internal standard (1-naphthoic acid, 1.4 mg/ml). This solution was then heated at 100°C for 2 h. Following hydrolysis of conjugates, the acids were extracted into dichloromethane (2 X 4 ml). The combined extract was dried (magnesium sulfate), transferred to a clean small culture tube and evaporated to dryness in a Savant Speed Vac Evaporator/Concentrator. The residue was derivatized as described in section 2.6.7. Saliva was obtained from a healthy human subject. The sample (0.5 ml) was diluted to 2 ml with deionized water to which was added 66 ul of perchloric acid and 30 ul of the internal standard solution (1-naphthoic acid, 1.4 mg/100 ml). The tube was centrifuged at 10,000 rpm for 15 min and the supernatant transferred to a large clean culture tube. The organic acids present were then extracted as described immediately above for the isolation of PAA from urine. Derivatization of the extracted organic acids is described in section 2.6.7.

2.6.5 Isolation of Ethylbenzene from Expired Air

Exhaled EBZ was isolated from rats following intraperitoneal administration of PLZ (15 mg/kg). The animals, after receiving the drug, were housed in glass metabolism cages and the expired air collected for 5.5 h as depicted in figure 5. Briefly, the expired air was evacuated from the metabolism cage, by vacuum (G), and drawn through molecular sieves (D) and into pentane (E) cooled to -32°C in a acetone/dry ice bath (F). In the pentane, the air was dispersed into fine bubbles using a piece of sintered glass attached to the bottom of the glass tube through which the air passed before entering the pentane. The air flow was monitored, and maintained at 800 ml/min, using an air flow meter (H). This rapid flow rate was required to provide adequate ventilation of the animal. Once sampling was complete, the pentane was evaporated, under a flow of nitrogen, to approximately 100 ul and 1 ul of this sample was injected into the GC/MS. The conditions used to separate the compounds present in the sample are given in table 1. For quantitative studies the internal standard deca-deuterated ethylbenzene (d_{10}-EBZ)(100 ug) was added to the pentane prior to the start of the collection period. Detection of the trapped ethylbenzene and d_{10}-EBZ was by GC/MS using selective ion monitoring. All samples were analyzed immediately after the collection period.

2.6.6 Derivatization of Phenelzine and Phenylethylamine

Each ethyl acetate extract obtained after extraction of the acetylated PLZ and PEA from section 2.6.1. was evaporated to dryness using a Savant Speed Vac Concentrator/Evaporator set at 45°C. To the dried residue was added 25 ul of dry ethyl acetate. From this point, samples analyzed for PLZ were treated differently. In the analysis of PLZ, PFPA (75 ul) was added and the mixture was heated at 65°C in a reacti-vial heating mantle. After 30 min, the reaction was stopped by adding 300 ul of cyclohexane followed by 0.5 ml of deionized water. The immiscible phases were gently mixed and 0.5 ml of 1N ammonium hydroxide was added to the tube. The phases were again gently mixed and separated by rapid centrifugation using a Dynac clinical centrifuge (2 min at Speed setting 100). The aqueous layer

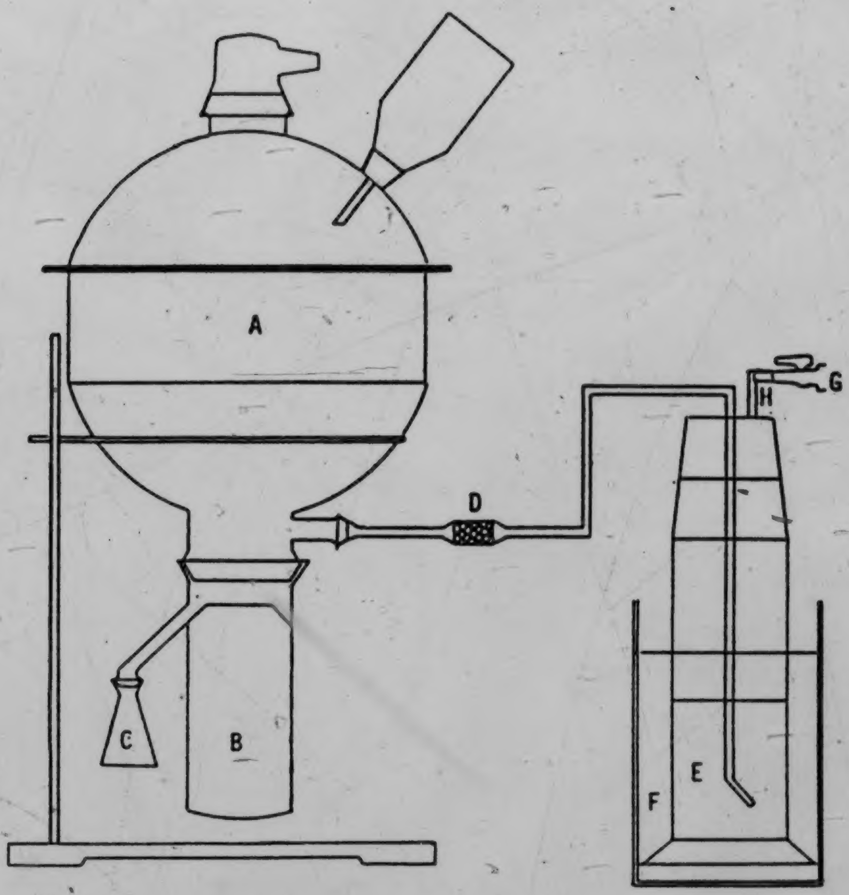


Figure 5. Illustrated above is the apparatus used to trap volatile compounds from the expired air of rats given an intraperitoneal dose of phenelzine. The major components are labelled and identified as: A. metabolic cage; B. feces trap; C. urine trap; D. moisture trap; E. pentane solvent; F. dry ice/acetone bath; G. vacuum; H. air flow meter.

was discarded and an additional 3 ml of a saturated sodium borate solution was added. The immiscible phases were mixed for 1 min on a Labquake® mixer, then separated by centrifugation of the sample for 4 min in a Dynac clinical centrifuge. The organic layer was transferred to a clean microfuge tube and evaporated to approximately 75 ul. Two microliters of this solution were injected into the GC column. Conditions used for the GC separation of the compounds present in the derivatized extract are described in table 1.

The PEA was derivatized by the procedure developed by Baker et al. (296). Briefly, to the extracted N-acetylphenylethylamine was added 25 ul of ethyl acetate and 75 ul of TFAA. The reaction was allowed to proceed for 30 min at room temperature. After 30 min, 300 ul of cyclohexane was added to the tube followed by 3 ml of sodium borate (saturated solution). The phases were gently mixed for 1 min in a Labquake® mixer and then separated by centrifugation in a Dynac clinical centrifuge. The top organic layer was transferred to clean 1.5 ml microfuge tubes. One microliter of the derivatized sample was injected into the GC. The conditions used for their GC separation are described in table 1.

Standard curves were generated by analyzing various quantities of PLZ and PEA by the same procedure. The standards were treated in a manner identical to the samples except a standard quantity of the compound was dissolved in deionized water.

2.6.7 Derivatization of Phenylacetic Acid

To the dried residue containing PAA isolated using the procedure descibed in section 2.6.4 was added 10 ul of TFAA and 100 ul of the derivatizing solution, 6 mg pentafluorobenzyl alcohol (PFBA) dissolved in 1 ml trifluoroacetic acid. The derivatization reaction was carried out in a teflon capped culture tube. The solution was heated to 85°C in a reacti-vial heating mantle. After 2 h the tube was removed, allowed to cool, and 300 ul of cyclohexane was added. The solvent was then evaporated to dryness under a stream of nitrogen. The remaining residue was redissolved in 300 ul of cyclohexane. One microliter of this solution was injected into the GC. The conditions used for the GC separation are again described in table 1. Standard curves of BA and PAA were generated by analyzing known quantities of these acids, dissolved in deionized water, by the procedure just described.

In addition several authentic organic acids and amino acids, dissolved in deionized water (1 ug/ml), were derivatized using this procedure. These acids were m-aminobenzoic acid, p-aminobenzoic acid, 1-phenylcyclopentanoic acid, D-alanine, D,L-phenylalanine, α-methyltyrosine, ibuprofen and o-methoxybenzoic acid.

2.6.8 Radiotracer Studies

Eight male Sprague-Dawley rats (200-250 g) were given intraperitoneal injections of phenelzine sulfate (15 mg/kg, calculated as the free base) each morning for 21 days. Body weights were determined immediately prior to the first injection and again on days 8, 15 and 21. A control group of 8 animals were treated in a similar manner with saline. On day 14, four animals of each group (PLZ treated or saline-treated control animals) were injected with PLZ sulfate (15 mg/kg, free base) spiked with 1-^{14}C-PLZ sulfate (2 uCi/animal). Following the injection, the animals were placed in metabolic cages (figure 5), and the urine, feces and expired air collected for the following 24 h. Animals were sacrificed after this 24 h period and their organs removed and weighed.

Expired radioactivity was also collected by drawing cage air, by vacuum, through 180 ml of an ethanolamine/2-methoxyflurane solution (1:2). The air flow was created using the in house vacuum system. A piece of sintered glass was used to disperse the drawn air into fine bubbles as it passed through the ethanolamine/2-methoxyflurane solution. Five milliliter portions of the trapping media were removed at 2, 4, 8, 16 and 24 h for liquid scintillation counting. The total volume of trapping medium was recorded prior to each collection to account for changes in the level of the solvents.

Aliquot portions of 24 h urine were assayed for total radioactivity. The urine (2 ml) was then separated into basic, neutral and unextracted fractions, by solvent extraction after appropriate pH adjustment (pH 2.0 and pH 8.0). PLZ and PEA were further isolated from the basic fraction, following derivatization with ethyl chloroformate, on a TLC plate (silica gel GF254). The procedure used for the isolation of these two compounds was as follows. To the urine sample was added enough sodium bicarbonate to make a saturated solution and the basified solution pH 8.0 was extracted with ethyl acetate (2 X 4 ml). The organic layers were combined and the sample was back extracted into 2.5 ml 0.5N HCl. The phases were mixed in a multitube vortex mixer and then separated by centrifugation in a Dynac clinical centrifuge (Speed setting 100). The aqueous layer was transferred to a clean large test tube. To this sample was added 100 ul ethyl chloroformate and excess sodium bicarbonate (enough to make a saturated solution). The samples were gently mixed for 30 min and extracted with 4 ml of ethyl acetate. Following mixing and centrifugation, the organic layer was retained and evaporated to dryness under a stream of nitrogen. The remaining residue was dissolved in 100 ul of ethyl acetate. A ten microliter sample was spotted on the silica gel TLC plate (10 cm X 10 cm) using a 10 ul Hamilton syringe for application of the sample. Once the sample was spotted, the plates were developed in 2 dimensions using cyclohexane/ethyl acetate (6:4) as the mobile phase. Appropriate zones, determined by co-chromatographing authentic PEA and PLZ derivatized in the same manner, were detected using short wave UV light, scraped off and transferred to plastic scintillation vials which contained 1 ml aqueous hydrofluoric acid. After the silica gel was dissolved, scintillation cocktail was added and the total ^{14}C-content determined by liquid scintillation counting.

To determine the level of radioactivity in selected organs, animals treated with radiolabelled PLZ were sacrificed 24 h following a dose of 1-^{14}C-PLZ sulfate. The lungs, liver, kidney, brain and spleen were removed and individually homogenized in isotonic KCl (40% homogenates, w/v). One milliliter of the resulting solution was removed and digested in 2 ml of protosol. Complete digestion required 2-4 wks, depending on the tissue. Once dissolved, the total quantity of ^{14}C-material was measured by liquid scintillation counting.

Samples were corrected for the amount of color quenching using the sample channel ratio method. In this procedure a known quantity of carbon tetrachloride was added to the scintillation cocktail along with a known quantity of ^{14}C-toluene. The ^{14}C-toluene was measured by liquid scintillation and the sample channel ratio and counting efficiency determined. A quench correction calibration graph was obtained by plotting the sample channel ratio versus the counting efficiency of each sample. This graph was then used to correct for color quenching when the ^{14}C-material in tissue samples was measured.

2.6.9 Monoamine Oxidase Inhibition

Animals treated with PLZ sulfate (15 mg/kg, free base), i.p., were sacrificed by cervical dislocation and the brain and liver quickly removed. The tissues were weighed and a 40% homogenate (w/v) made with cold isotonic KCl. After the tissue were thoroughly homogenized, using a teflon tissue homogenizer, the tissue homogenates were stored at -26°C until the enzyme activity was measured.

On the day of analysis the tissue samples were thawed. To the appropriate number of tubes was added 250 ul of 0.5M phosphate buffer (pH 7.4). The tubes were then placed on ice. Twenty-five microliters of the homogenized tissue sample was then added to each tube. For blanks, isotonic KCl was used in place of the tissue. Following addition of the tissue, 25 ul of ^{14}C-5-hydroxytryptamine or ^{14}C-phenylethylamine solutions (section 2.9) were added to the appropriate tubes which were incubated for 20 min at 37°C. During incubation, solutions were gently mixed in a metabolic shaker (200 rpm). After 20 min the reaction was stopped by placing the tubes on ice and adding 2N HCl (200 ul). The radiolabelled 5-hydroxyindoleacetic acid or PAA, formed by the action of MAO on ^{14}C-5-hydroxytryptophan and ^{14}C-phenylethylamine, respectively, were extracted into toluene (6 ml). The extraction was accomplished by vortexing the tubes in a multitube vortex mixer for 3 min and then centrifuging the sample for 5 min in a Dynac clinical centrifuge. Following separation, the aqueous layer was frozen at -26°C for 30 min. The liquid toluene layer was then poured into 9 ml of the liquid scintillation cocktail and the quantity of the radiolabelled acid present measured by liquid scintillation. All radioactivity was measured over a 10 min period. The activity of the MAO A and B enzyme was positively correlated to the quantity of 5-hydroxyindoleacetic acid or PAA formed, respectively.

The MAO activity was measured in rats chronically treated with PLZ sulfate (15 mg/kg, free base) for a 21 day period. On days 1, 3, 5, 7, 9, 12, 15, 18, and 21 the enzyme assay procedure was carried out on brain and liver tissue and the MAO activity in these two organs determined. The animals were sacrificed 6 h after the dose of PLZ. Control animals were treated with saline for the same periods.

2.6.10 Isolated Rat Liver Perfusion Studies

The isolated rat liver perfusion was performed according to the procedure of Tam et al. (52). The apparatus used is depicted in figure 6. Male Sprague-Dawley rats (200-250 g) were used in these experiments. These animals were anesthetized with diethyl ether during the surgical procedure. The abdomen was opened and the portal vein as well as both the upper and lower vena cava veins were cannulated using intramedic PE-50 (portal vein), PE-100 (upper vena cava) and PE-10 (lower vena cava) tubing. Effluent obtained from the lower vena cava cannula was used to monitor oxygen consumption. Following cannulation, the liver was isolated from the animal and placed on the liver platform. The liver was perfused through the portal vein cannula with oxygenated (95% O_2/5%CO_2) Kreb's bicarbonate buffer (pH 7.4) supplemented with 2 g/L dextrose. During the experiment the pH of the perfusion buffer was continuously monitored and adjusted to pH 7.4 using an automatic titration system. The perfusion flow through the liver was achieved by gravity and was dependent upon the resistance in the liver vasculature. Flow rates between 20 and 30 ml/min were used for experiments. The viability of the liver was monitored by examining the appearance of the liver and measuring oxygen consumption and pH. Livers used in these experiments were maintained viable for a 2 h period. The metabolism studies utilized the "once through" perfusion method. PLZ, dissolved in Kreb's bicarbonate buffer, was introduced into the perfusion buffer before entering the liver using a constant rate infusion pump. The inlet concentration was determined by withdrawing a

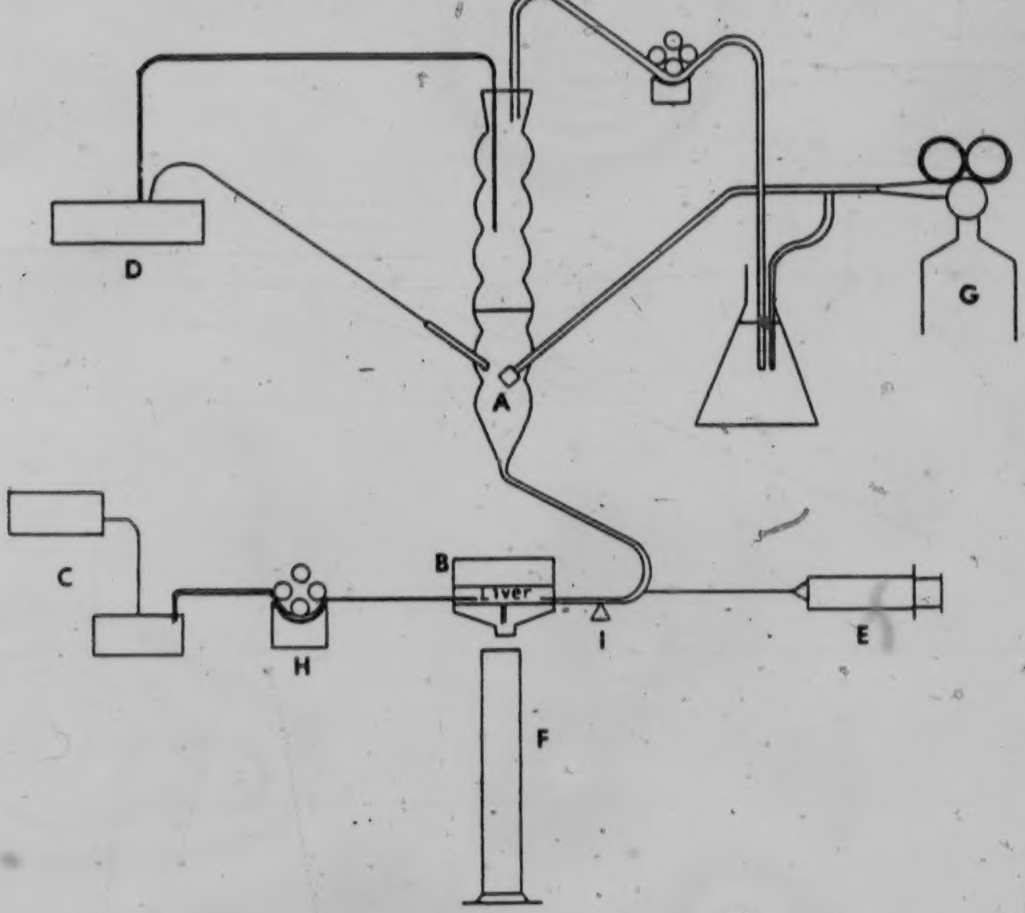


Figure 6. This figure illustrates the apparatus used for the isolated perfused rat liver studies. The important features of the system are labelled and identified as follows: A-buffer reservoir; B-liver platform; C-oxygen meter; D-automatic pH titration unit; E-Harvard Infusion pump; F-effluent collection; G-O_2/CO_2 (95:5) tank; H-peristaltic pump used to withdraw samples to measure O_2 content; I-sampling site used to withdraw the drug solution to measure the Cin concentration.

sample of perfusate just prior to entering the liver. Samples of the liver effluent, obtained from the upper vena cava cannula, were obtained at 0, 1, 3, 5, 7, 9, 12, 15, 18, 21, 24, 27, 30, 35, 40, 45, 50, 55 and 60 min, after the start of infusion. PLZ and PEA in the effluent were analyzed by the procedure described in section 2.6.3, while PAA present was analyzed by the procedure described in section 2.6.4.

2.6.11 *In Vivo* Animal Experiments

During experiments, animals were housed in clear plexi-glass cages containing wood chips as adsorbant. Free access to both food and water was allowed. Standard rat pellets were provided as food. The animals were on a 12 h day/12 h night cycle.

2.6.11.1 Jugular Vein Cannulation

Jugular vein cannulation was performed according to the method of Upton et al. (268), with minor modifications. The jugular vein was cannulated with silastic tubing (0.025 cm i.d. X 0.047 cm o.d.) attached to a length of PE-50 tubing. Only the Silastic® tubing was inserted (33 mm) into the jugular vein. The cannula was secured in the vein with a stay suture at the point of insertion into the jugular vein. The end of the silastic tubing was cut perpendicular to its length to prevent the cannula from adhering to the side of the vein during collection of the blood sample. The PE-50 tubing was passed subcutaneously under the skin and exteriorized behind the ear. Approximately 3 cm of the cannula was exposed. The cannula was filled with a 4% sodium citrate solution as anticoagulant while the animal recovered from surgery and again following the completion of experiments. This solution was changed daily. When not used, the cannula was plugged with a 5 mm length of 26 gauge stainless steel wire. This cannula was used for both drug administration and blood sampling. At the start of the experiment the exposed cannula was attached to approximately 20 cm of PE-50 tubing using a 26 gauge needle with the hub removed, as a joint. This tubing was allowed to hang outside the cage. To dose the animal, the sodium citrate solution was removed, and a 300 ul blank blood sample withdrawn. The drug dissolved in 0.2 ml phosphate buffered saline (PBS) (Section 2.9) was then administered. This solution was washed into the rat with 0.4 ml PBS. Samples were obtained at 5, 10, 15, 20, 30, 60, 100, 180, 260, 320, 400 min post-injection. The sampling procedure was carried out as follows. The PBS was removed and blood (100-500 ul) was drawn into a 1 ml syringe. The blood remaining in the cannula was flushed back into the animal with saline. The volume of blood removed was replaced with an equal volume of PBS. After the experiment, the cannula was once again filled with 4% sodium citrate. All solutions used in the experiment, except the drug solution, were sterilized by autoclaving. The solution of PLZ prepared for injection was filter sterilized through 0.22 um millipore filters.

2.6.11.2 Biliary Excretion Studies

Animals utilized for biliary excretion studies had both their bile duct and jugular vein cannulated. The jugular vein cannulation was carried out as described in section 2.6.11.1. The bile duct cannulation method is illustrated in figure 7. Male Spague-Dawley rats (400-500 g) were anesthetized with diethyl ether, the abdominal cavity opened and the intestinal tract moved such that the bile duct was exposed. Moistened gauze was placed over the intestine to prevent drying. A small incision was made in the bile duct approximately 4 cm below the hilus of the liver. The re-entrant bile duct cannula was inserted 4-5 cm in a proximal direction and secured in place with 3-0 silk thread. This cannula was looped under the upper lobe of the liver and secured to the abdominal wall with a stay suture. A second incision in the bile duct was made 0.5 cm dorsal to the first incision. A second cannula (PE-10) was inserted into this incision (1 cm) in the direction of the liver. Again the cannula was secured into place with 3-0 silk thread. This cannula was looped proximal to the right kidney and secured to the roof of the abdominal wall at several places with stay sutures. A small incision was made in the side of the abdominal wall and the two cannula passed between the muscle and skin to an exit point behind the ear. The two cannulae were secured to the skin behind the ear with a stay suture. The two

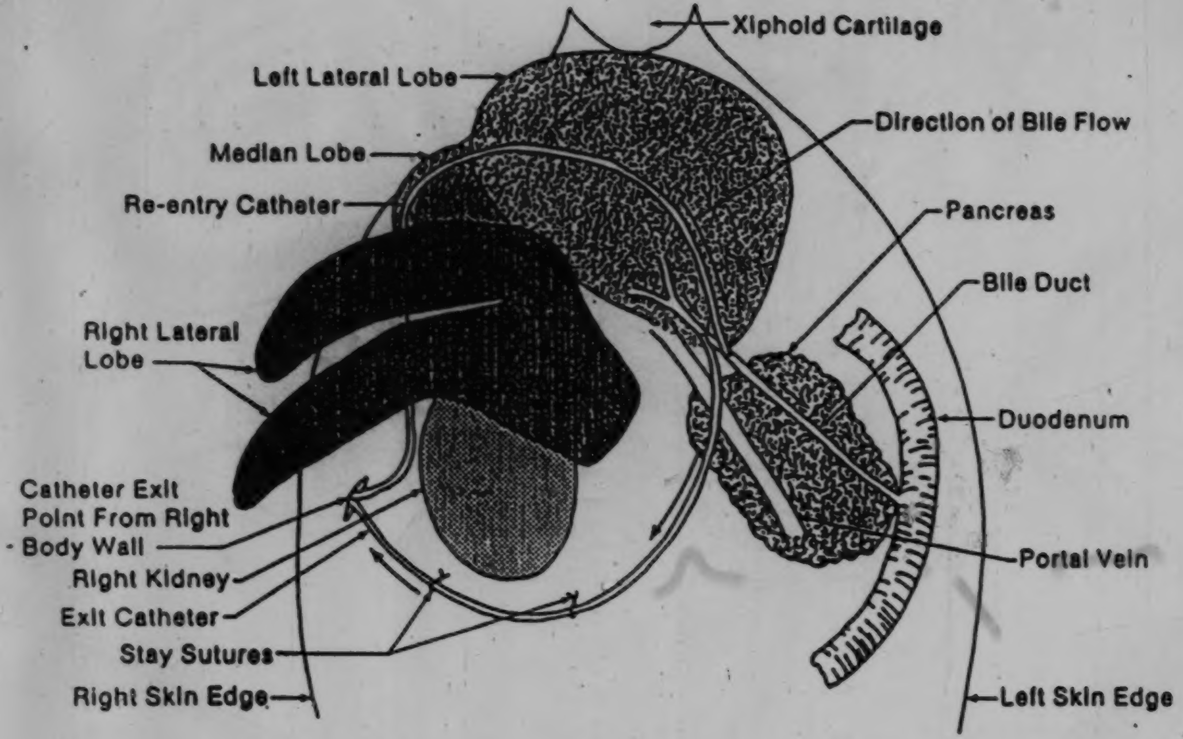


Figure 7. Ventral view of the abdomen of a rat showing the bile duct with the exit an re-entry catheters installed. Landmark structures are included for reference.

exposed pieces of cannula were joined using a 5 cm length of Silastic® tubing (0.025 cm i.d.) Following the cannulation procedure, all incisions were closed using Ti-cron® thread. After recovery from surgery, rats were given 5 ml of a 10% travesol solution orally to stimulate peristalsis. The bile duct-cannulated rats were maintained in conventional cages, with free access to food and water, until required for experiments.

During sampling, the animals were placed in a harness which contained a small sampling vial (Fig 8). The harness was situated directly behind the front legs. The sampling bottle was attached to the bile duct cannula and bile was collected during the 7 h experimental period. Lost fluid was replaced with an equal volume of PBS, administered through the jugular vein cannula. After the experiment, the two bile duct cannulae (exit and re-entry) were re-connected to establish recyling of bile.

2.7 Lidocaine Experimental

2.7.1 Analysis of the Phenolic Metabolites of Lidocaine

2.7.1.1 Acetylation

The phenolic metabolites of LID were isolated from 24 h urine following acid hydrolysis, achieved by adding 0.5 ml of 6N HCl to 0.5 ml of urine and heating the solution for 15 min at 100°C. The cooled solution was neutralized with 1 N sodium hydroxide then basified by adding excess potassium bicarbonate such that solid potassium bicarbonate remained at the bottom of the tube after mixing. To this solution was added 300 ul of acetic anhydride. Once the effervescence stopped, the derivatives formed were extracted into ethyl acetate (1 X 4 ml). The organic layer was transferred to a clean small culture tube and evaporated to dryness in a Savant Speed Vac Evaporator/Concentrator. The dried residue was reconstituted into 100 ul of ethyl acetate. One microliter of the extract was injected into the GC and the compounds present separated using the conditions described in table 1.

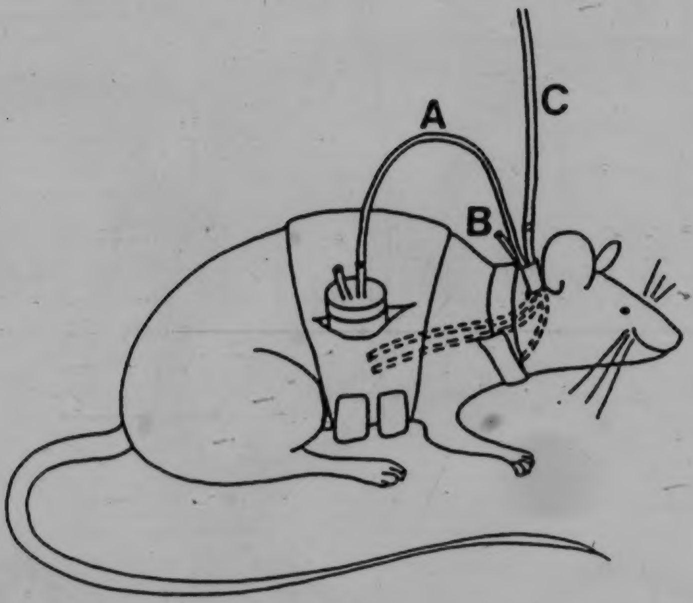


Figure 8 Exterior of rat showing harness for bile collection and positions of catheters.

A Exit catheter attached to collection vial.

B Re-entry catheter (not connected during bile collection)

C Jugular catheter with extension for remote blood collection.

2.7.1.2 Pentafluorobenzoylation

Conjugates present in the urine sample (100 ul-500 ul) were acid hydrolyzed as in the procedure used for the acetylation of these compounds. Once these conjugates were hydrolyzed the solution was neutralized with 3 N sodium hydroxide then basified with potassium bicarbonate (200 mg/sample). To this basic solution was added 5 ul of PFBC and 3 ml toluene. The immiscible phases were mixed for 30 min in a multitube vortex mixer and then centrifuged in a Dynac bench top centrifuge for 5 min. The organic layer was transferred to a clean small culture tube and evaporated to dryness using the Savant Speed Vac Evaporator/Concentrator. To the dried residue was added 300 ul toluene and 600 ul 1% ammonium hydroxide (v/v). The phases were mixed for 30 sec and then transferred to a 1.5 ml microfuge tube. The sample was then centrifuged briefly in a Microfuge B centrifuge. The top organic layer was retained and transferred to a clean 1.5 ml microfuge tube. One microliter of this sample was injected into the GC. The conditions used for separation are described in table 1.

2.7.1.3 Trifluoroacetylation

To 2 ml of urine or water containing lidocaine or one of its metabolites was added enough sodium carbonate to make a saturated solution (pH 9.0). To this solution was added 4 ml ethyl acetate. The immiscible phases were mixed for 10 min in a multitube vortex and then centrifuged for 10 min in a Dynac bench top centrifuge. The organic layer was transferred to a clean small test tube. The extraction procedure was repeated and the organic layers combined. The organic layer was evaporated to dryness in a Savant Speed Vac Evaporator/Concentrator. To the dried residue was added 25 ul ethyl acetate and 75 ul TFAA. The reaction was allowed to proceed for 30 min at room temperature. After 30 min 300 ul of cyclohexane and 3 ml of a saturated sodium borate solution was added. The immiscible phases were gently mixed for 1 min and then centrifuged for 1 min. The organic layer was transferred to a clean dry test tube. One microliter of this solution was injected into the GC or MS.

2.7.2 Isolation of the Glucuronide of 4-Hydroxyxylidine

Urine samples (2 ml), obtained from patients who had received LID, were basified to pH 9.0 with 1N sodium hydroxide. The basified urine sample was then extracted once with 4 ml of ethyl acetate. The phases were mixed for 5 min in a multitube vortex mixer then centrifuged in a Dynac bench top centrifuge (Speed setting 100) for 5 min. The organic layer was discarded. The aqueous layer was acidified to pH 1.0 with 1 N HCl and once again extracted, as above, with 4 ml ethyl acetate. The organic layer was again discarded and the retained aqueous layer evaporated to dryness using a Savant Speed Vac Evaporator/Concentrator at 45°C. The resulting residue was taken up in 100 ul of freshly distilled dimethyl sulfoxide and kept under a nitrogen atmosphere, until derivatized according to the procedure described in section 2.7.3.

2.7.3 Derivatization of 4-Hydroxyxylidine Glucuronide

The procedure used to derivatize the glucuronide conjugate of 4-OH-Xyl was essentially the same as that described by Thompson et al. (132). Briefly, the residue obtained by evaporating the urine sample by the procedure described in section 2.7.2, was dissolved in 100 ul dimethyl sulfoxide (freshly distilled). To this solution was added 25 ul of the dimethylsulfinylmethide carbanion solution (section 2.9) and the mixture was left standing at room temperature for exactly 15 min. After this time 10 ul of methyl iodide was added. The reaction was allowed to proceed for a 1.5 h period before 1 ml of water was added to stop the reaction. The derivatives formed were extracted into 1 ml of chloroform by mixing the immiscible phases for 10 min followed by a 10 min centrifugation. The organic layer was transferred to a clean test tube, dried with magnesium sulfate, and evaporated to 25 ul under a stream of nitrogen. Two microliters of this solution were injected into the GC/MS. The conditions used for the chromatographic procedure are described in Table 1.

2.7.4 Collection of Human Urine

Urine samples were obtained from patients who had received an intravenous infusion of LID (2 mg/min). The samples of urine were collected in large plastic containers during the drug infusion period.

2.8 Doxapram Experimental

2.8.1 Analysis of Doxapram

Urine (2-5 ul) and plasma (20-100 ul) were made up to a final volume of 200 ul with deionized water in 1.5 ml microfuge tubes. To these samples was added 25 ul of the internal standard solution (diazepam.HCl, 0.11 mg/ml) and 7 ul of concentrated perchloric acid. The precipitated protein was removed by centrifugation at 8730Xg in a Microfuge B® for 4 min. The supernatant was transferred to a clean microfuge tube and the perchloric acid neutralized with solid potassium bicarbonate. The potassium perchlorate which formed was removed by centrifuging the sample for 4 min. The supernatant was again transferred to a clean microfuge tube to which was added enough solid potassium bicarbonate to make a saturated solution. Following the addition of 1 ml of ethyl acetate the immiscible phases were mixed by vortexing the sample for 5 min in a multi-tube vortex apparatus followed by centrifugation for 4 min. The organic layer was retained and evaporated to dryness in a Savant Speed Vac Evaporator/ Concentrator. To the residue was added 50 ul toluene. Two microliters of this extract was injected into the GC. Separation of the compounds present was achieved using the conditions described in table 1.

2.8.2 Human Experiments

Infants who had received constant intravenous infusion (1-2.5 mg/kg/h) of DOX.HCl were used in the DOX study. Blood samples (300-500 ul) were withdrawn by a heel prick and placed into plastic polyethylene tubes, containing ethylenediaminetetraacetic acid as anticoagulant. The plasma was isolated by centrifugation and then stored at -26°C until analyzed. Total body clearance (ClTB) and renal clearance (Clr) of DOX were evaluated for these infants. The clearance studies were designed so that; two steady state blood samples were obtained 6 h apart. All the urine excreted during this 6 h time period was also collected. Half-life (T1/2) measurements were made by measuring the DOX concentration in 5 samples, consecutively withdrawn from these infants at 2 h intervals; once infusion was stopped. The value of β was determined using a HP-31 linear regression program. Using β the half-life was then calculated according to the formula in appendix B.

2.9 Solutions Used

The solutions described below were used in several experiments.

1. Phosphate Buffered Saline (PBS)

   NaCl 40 g, KCl 1 g, $Na_2HPO_4.7H_2O$ 10.8 g, KH_2PO_4 1.0 g, H_2O to 5 l. pH 7.4.

2. Dimethylsulfinylmethide carbanion

   Five millimoles NaH, rinsed three times with diethyl ether, was added to 5 ml of dry dimethyl sulfoxide. The mixture was heated to 50°C, under N_2, until the evolution of hydrogen stopped. This solution was stored at 4°C under N_2.

3. ^{14}C-Phenylethylamine

   ^{14}C-Phenylethylamine (0.1 mCi/ml)-25.2 ul

   Cold phenylethylamine (6.3 mg salt/100 ml)-302.4 ul

   Distilled water - 672.4 ul

4. ^{14}C-5-Hydroxytryptamine

   ^{14}C-5-Hydroxytryptamine (0.1 mCi/ml)-20 ul

   Cold 5-hydroxytryptamine (7.7 mg salt/100 ml)-525.2 ul

   Distilled water - 455.2 ul.

in the increased production of toxic metabolites, serious side effects may be observed during drug therapy.

Essential in the study of drug metabolism is the development of appropriate analytical techniques for the isolation and identification of metabolites. Knowledge in the area of drug metabolism often parallels new technologies in analytical chemistry. In this study TLC, GC and GC/MS were all utilized

3.1 Studies Conducted on Phenelzine

3.1.1 Analysis of Phenelzine and Phenylethylamine

Several methods have been described for the analysis of PLZ in biological fluids. These methods, however, require sophisticated instrumentation (41), lack sensitivity (162) or are nonspecific (42). Frequently mass spectrometry is used for the analysis of PLZ in biological samples (41,167). However, this instrument is not routinely available to many investigators. GC, linked to either an ECD or NPD, has also been utilized for the analysis of PLZ in biological fluids (174,44). To enhance its stability and detectability, PLZ is frequently derivatized prior to its analysis. Reagents most commonly used for PLZ derivatization possess a carbonyl functional group. These compounds react with PLZ through a condensation-type reaction forming the corresponding hydrazone (44,41). However, these reagents are not useful for the detection of PLZ metabolites. Only those compounds which retain the NH_2 group of the hydrazine in an unaltered form will react with aldehydes or ketones in this manner. In this study a method which was capable of derivatizing PLZ, PEA as well as other possible metabolites was sought. The reaction of amines with aldehydes give Schiff's bases which are generally not stable enough for subsequent GC analysis. For this reason, condensation-type derivatizing reagents were not investigated. Cooper et al. (174) described a method of derivatizing PLZ using the perfluoroacylating reagent, HFBA. Attempts to utilize this method failed. Numerous peaks were detected following GC analysis of the reaction mixture, even when authentic PLZ was derivatized by this procedure. In view of the limitations of previous methods, a superior procedure for the analysis of this compound in biological material was required. A gas chromatographic procedure, providing both sensitivity and specificity, was envisaged.

For the GC analysis of PLZ at low concentrations, derivatization prior to chromatographic separation is required. Low level detection of underivatized amines is compromised by surface adsorption to the GC column and/or thermal decomposition during the chromatographic procedure (298). GC separation of PLZ, a hydrazine derivative, would be expected to behave in a manner similar to underivatized amines. In this study the derivatization procedures investigated included acylation techniques which would derivatize metabolically produced phenolic OH groups, as well as amine functionalities (primary and secondary) in aqueous solutions.

The derivatization of PLZ and PEA, a metabolite of PLZ, with ethyl chloroformate, was investigated (Fig 9). This reagent is known to react quantitatively with amine and phenolic amine compounds, including PEA, in alkaline solution (299). Ethyl chloroformate has not been used previously to derivatize hydrazine type compounds. The identities of the ethyl chloroformate-derivatized PLZ and PEA products were confirmed by mass spectral analysis (Fig 9). PLZ formed mainly a mono-derivatized species 13 with ethyl chloroformate, although a di-derivatized 14 compound was also detected. Synthetically prepared N'-acetyl-PLZ 18, a postulated metabolite of PLZ, also formed a mono- 15 and di-derivatized species 16 with this reagent (Fig 10). Detection of the carbamate derivatives of PLZ and PEA 17 was achieved using a NPD following their GC separation (Fig 11). This analytical procedure was used for the analysis of these compounds in perfusates obtained from an isolated rat liver infused with a solution of PLZ. However, the method proved inadequate, lacking sufficient sensitivity for the analysis of PLZ or PEA in blood withdrawn from a rat that had received PLZ (15 mg/kg,free base). Detection below 100 ng of PLZ per sample was difficult to achieve.

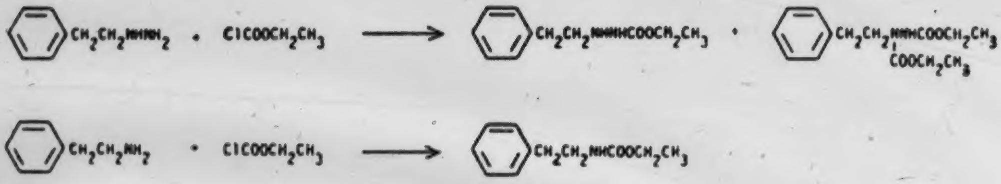
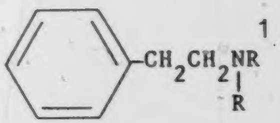


Figure 9. The derivatives formed following the reaction of phenelzine and phenylethylamine with ethyl chloroformate in an alkaline solution.

Figure 10. The major electron-impact fragment ions of phenelzine, 2-phenylethylamine and N'-acetylphenelzine derivatized with either ethyl chloroformate or acetic anhydride.



	R	R^1	m/z (% relative abundance)
13	H	$NHCOOCH_2CH_3$	208(-) 176(38) 117(85) 105(33) 104(26) 91(70) 89(100) 71(86)
14	$COOCH_2CH_3$	$NHCOOCH_2CH_3$	280(-) 189(63) 176(55) 117(73) 104(99) 91(74) 89(68) 71(100)
15	$COOCH_2CH_3$	$NHCOCH_3$	250(-) 159(6) 146(33) 117(19) 104(65) 91(47) 87(100) 71(12)
16	$COOCH_2CH_3$	$NCOCH_3$ $COOCH_2CH_3$	322(-) 189(19) 176(43) 117(94) 104(100) 91(68) 89(65) 87(83) 71(50)
17	H	$COOCH_2CH_3$	193(12) 104(43) 102(100) 91(90) 65(31)
18	H	$NHCOCH_3$	178(-) 91(10) 87(56) 45(100) 43(43)
19	$COCH_3$	$NHCOCH_3$	220(-) 117(22) 104(14) 91(19) 87(100) 45(21) 43(40)

To permit the analysis of PLZ and PEA in blood samples, other derivatization procedures were investigated. Numerous fluorinated derivatizing reagents are available for reaction with amine (primary and secondary) and OH functional groups. These reagents are commonly used for the low level analysis of a variety of compounds (300,301). The fluorine atoms render the derivatives sensitive to an ECD. This detector is capable of responding to as little as 1 pg of compound passing through the detector cell. Besides the enhanced detectability provided by an ECD, fluorinated reagents increase the volatility of the derivatized compound. The increased volatility also improves detector response to these derivatized compounds; sharp and narrow GC peaks are generally obtained. PFBC and pentafluorobenzenesulfonyl chloride, common acylating reagents used to derivatize amine and phenolic compounds were evaluated (221,222). Following the derivatization of PLZ with either of these reagents, GC peaks were observed which possessed the same retention times as PEA similarly derivatized. These derivatives of PLZ and PEA could not be separated by varying GC conditions or by changing the column stationary phase. The products obtained following the reaction of PLZ with either of these reagents have not been identified, but could conceivably result from the breakdown of PLZ to PEA during the derivatization procedure. The PEA formed then reacted with the derivatizing reagent. The actual percentage of PLZ converted to the proposed product was quite small, but quantities were sufficient to interfere with the trace analysis of PEA isolated from the blood of rats treated with PLZ. Mass spectrometry studies could not be carried out on the decomposition product, since the quantity formed was to small. The amount of PEA present in the blood of these animals was significantly overestimated using this procedure.

Cooper et al. (174) observed that PLZ was unstable in basic solutions. To overcome this instability and to enhance the extractability of PLZ, aqueous acetylation (i.e. derivatization of PLZ in aqueous solution) with acetic anhydride followed by extraction into a water immiscible organic solvent was investigated. The conditions used were similar to those used previously for the aqueous acetylation of amino and aminophenolic compounds (302,303). Previous studies have demonstrated that acetylation of PLZ could yield three products (Fig

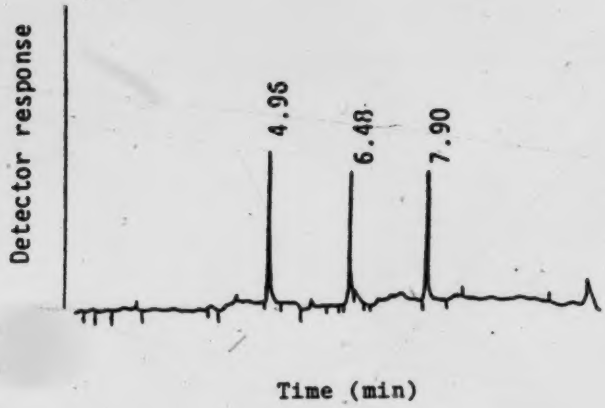
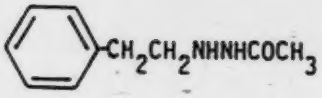


Figure 11. The GC trace of ethyl chloroformate-derivatized phenelzine (7.90 min), phenylethylamine (4.96 min) and the internal standard p-chlorophenylethylamine (6.48 min). These derivatives were isolated from the effluent obtained by perfusing an isolated rat liver with phenelzine dissolved in Kreb's bicarbonate buffer. Detection of these carbamate derivatives was achieved using a nitrogen-phosphorus detector. The concentration of phenelzine and phenylethylamine in this sample was 1.16 ug/sample and 0.58ug/sample, respectively.

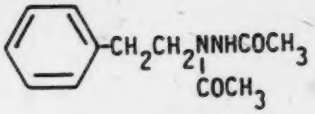
12), depending on the derivatization conditions used (175,304). Treatment of PLZ with acetyl chloride or acetic anhydride, under anhydrous conditions, produced a mixture of mono-derivatized compounds, whereas aqueous acetylation of PLZ with acetic anhydride was thought to form N,N'-diacetyl-PLZ 19 (Fig 12). In the present study it was imperative to confirm the true identity of the PLZ derivative formed during aqueous acetylation. Both PLZ and synthetically prepared N'-acetyl-PLZ yielded the same product (GC and GC/MS evidence) following aqueous acetylation. This product 19 could not be further derivatized with ethyl chloroformate. In contrast, reaction of N'-acetyl-PLZ with ethyl chloroformate produced a product identified as N'-acetyl-N-ethoxycarbonylphenelzine 15 (Fig 10). From these results it was concluded that PLZ almost exclusively forms the di-acetylated product 19 during derivatization with acetic anhydride, under aqueous conditions.

Further derivatization of di-acetylated PLZ was investigated using several perfluoroacylating reagents. It has been demonstrated that aryl and aliphatic amides, formed during the aqueous acetylation of the corresponding amine, could be further derivatized under anhydrous condition with TFAA or PFPA. The reaction produced diacyl derivatives sensitive to an ECD (182,302). PFPA reacted with N,N'-diacetyl-PLZ, producing two products, which are believed to be formed by a PFPA-catalyzed cyclization of N,N'-diacetylphenelzine. The cyclization reaction is thought to produce a pyrazole ring. The proposed structure of these two derivatives and their corresponding EI/ and CI/MS fragmentation pattern are depicted in figure 13. Treatment of the internal standard benzylhydrazine (aqueous acetylated) with PFPA also produced two derivatives which could be separated by GC. Derivatization of N,N'-diacetyl-PLZ 19 with other perfluoroacylating reagents was also attempted. Heptafluorobutyric anhydride (HFBA) reacted with N,N'- diacetyl-PLZ, producing two separable products, while derivatization of N,N'-diacetyl-PLZ with TFAA produced a number of products (GC evidence).

The fact that PLZ formed two derivatives, when reacted with PFPA, did not limit the usefulness of this technique for the quantitative analysis of PLZ. Calibration graphs were

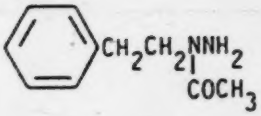


18



$C_6H_5CH_2CH_2N(COCH_3)NHCOCH_3$

19



$C_6H_5CH_2CH_2N(COCH_3)NH_2$

20

Figure 12. The possible structures of the product(s) formed following the reaction of phenelzine with either acetic anhydride or acetyl chloride. The identities are N'-acetylphenelzine 18, N,N'-diacetylphenelzine 19 and N-acetylphenelzine 20.

generated by subjecting varying quantities of PLZ (0-9 ug) to the same analytical procedure (acetic anhydride/PFPA). The GC peaks of the two derivatives were integrated and summed for each concentration of PLZ, then divided by the summed peak areas of similarly derivatized benzylhydrazine. When these ratios were plotted versus the corresponding quantities of PLZ, a linear relationship was always obtained (Fig 14). The slope (0.15 ± 0.02, n=11) of standard curves, generated in this manner on different days, varied only slightly. The coefficiant of correlation was consistently 0.99. The coefficiant of variance was calculated to be 7.5% when blank blood samples spiked with PLZ (2.0 ug) were analyzed in triplicate. New standard curves were routinely generated for each set of blood samples analyzed. Typical GC traces of PLZ, isolated from blood or a spiked water sample and derivatized with acetic anhydride/ PFPA, are depicted in figures 15-16. Acetic anhydride/PFPA derivatized extracts of blank blood and bile are shown in figures 17-18. The limit of detection of PLZ by this method was 10 ng/ml of sample. If present, N-acetylphenelzine could interfere with this assay; however, it is believed from previous studies conducted by other researchers (167) that this compound is not a significant metabolite of PLZ. To confirm that N'-acetylphenelzine 18 did not interfere with the analysis of PLZ, blank blood samples (500 ul) were spiked with N'-acetylphenelzine (10 ug) and then analyzed using this analytical procedure. No GC peak corresponding to derivatized PLZ was observed, indicating that N'-acetylphenelzine could not be recovered from the blood sample. This difficulty in recovering N'-acetylphenelzine from blood samples has been previously demonstrated (125).

PEA could not be analyzed by this procedure because of interfering GC peaks. This compound was analyzed in blood as the N-acetyl-N-trifluoroacetyl derivative using the procedure described by Baker et al. (296). The derivative formed was identified by its sensitivity to electron capture detection and comparison of its electron impact mass spectral fragmentation pattern to a previously published mass spectrum of this derivatized compound (219). PLZ did not interfere with the analysis of PEA using this procedure.

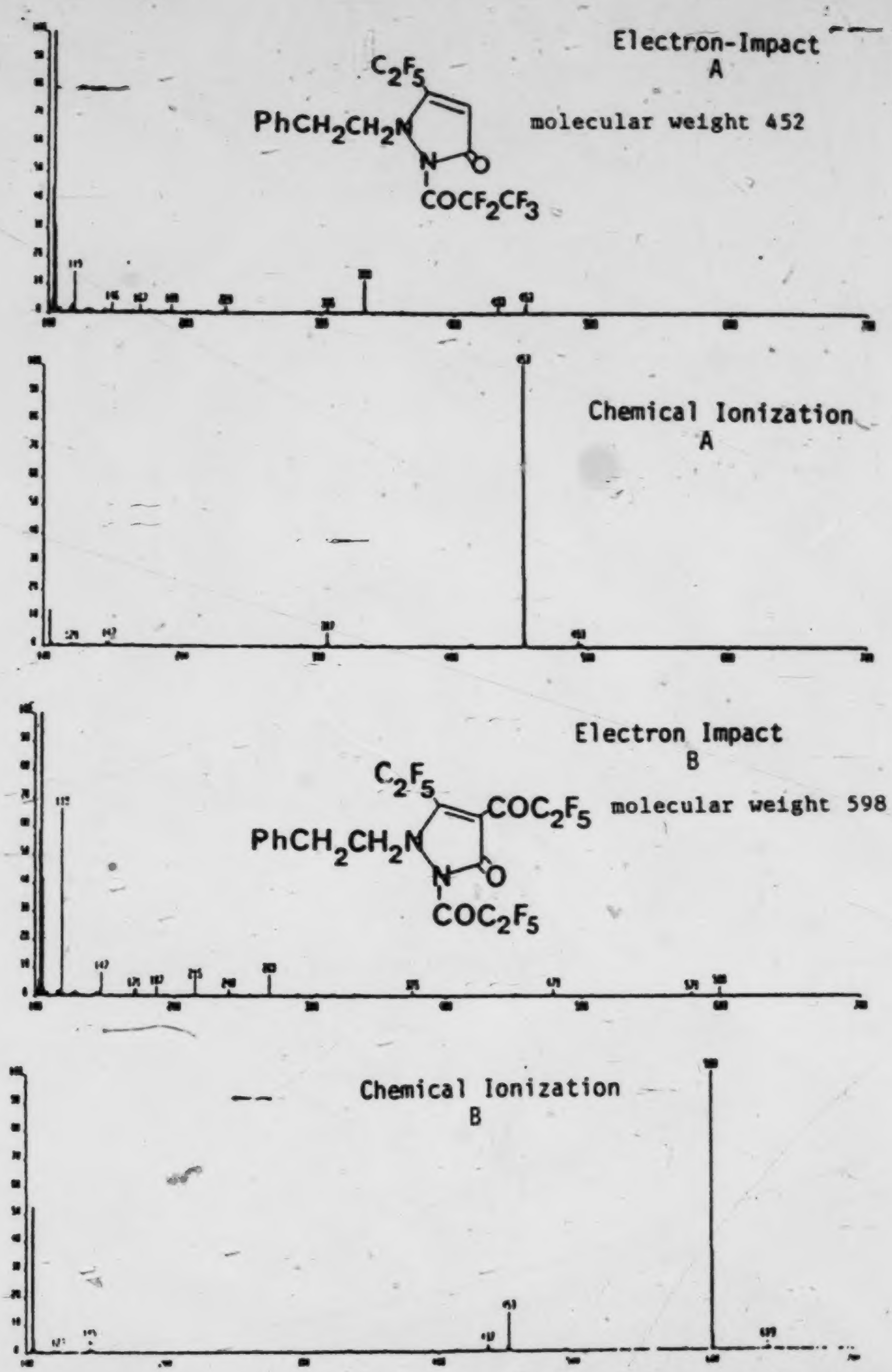


Figure 13. The proposed structure and corresponding EI and CI mass spectra of the two derivatives (A and B) formed when phenelzine was reacted with acetic anhydride and pentafluoropropionic anhydride.

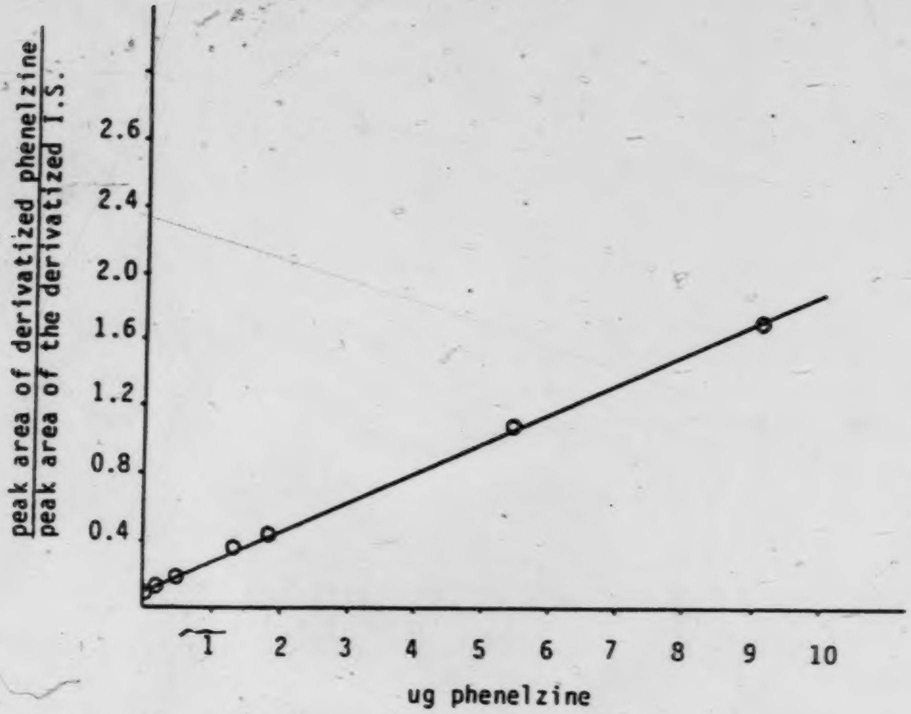


Figure 14. The calibration graph of phenelzine, 0-9 ug/sample, derivatized with acetic anhydride and pentafluoropropionic anhydride. The total volume of these samples was 2 ml. The conditions used are given in the text. Benzylhydrazine (3 ug/sample) was used as internal standard.

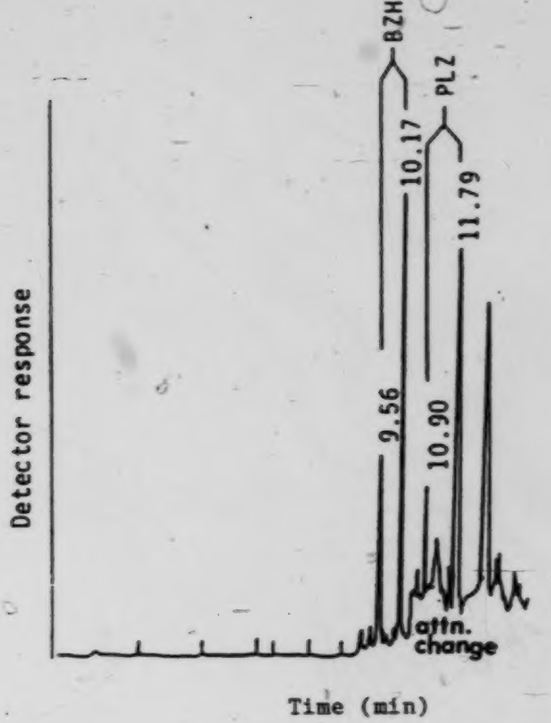


Figure 15. The GC trace of the acetic anhydride/pentafluoropropionic anhydride derivatized extract of blood (400 ul) obtained from a rat that had received a dose of phenelzine (15 mg/kg, free base). The amount of phenelzine present in this sample was calculated to be 0.40 ug. The internal standard benzylhydrazine (3.0 ug) was added to the sample prior to analysis. GC peaks are identified as above; BZH=acetic anhydride/PFPA derivatized benzylhydrazine and PLZ=acetic anhydride/PFPA derivatized phenelzine. The other peaks present in the GC trace were not identified.

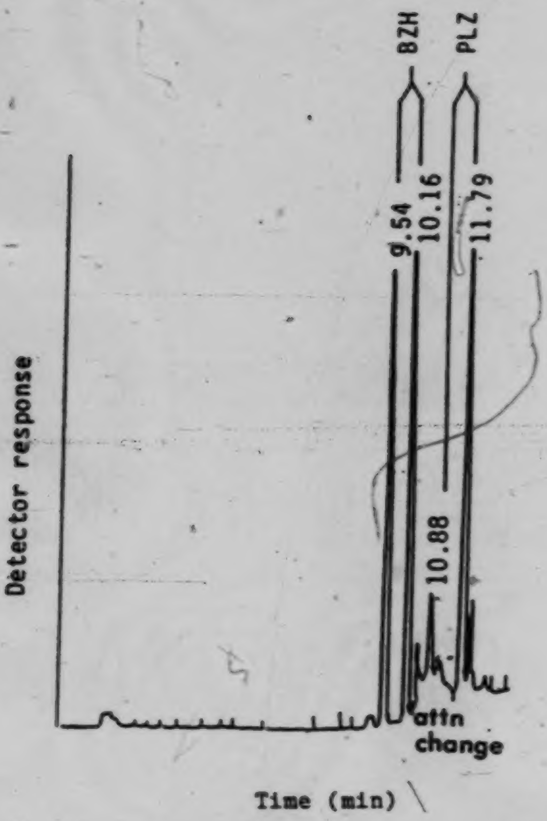


Figure 16. The GC trace of a derivatized extract of water (2 ml), spiked with phenelzine (17 ng). Benzylhydrazine (3.0 ug/sample) was also added to the water sample prior to analysis. The labelled GC peaks were identified by EI/MS; PLZ=acetic anhydride/PFPA derivatized PLZ, and BZH=acetic anhydride/PFPA derivatized benzylhydrazine.

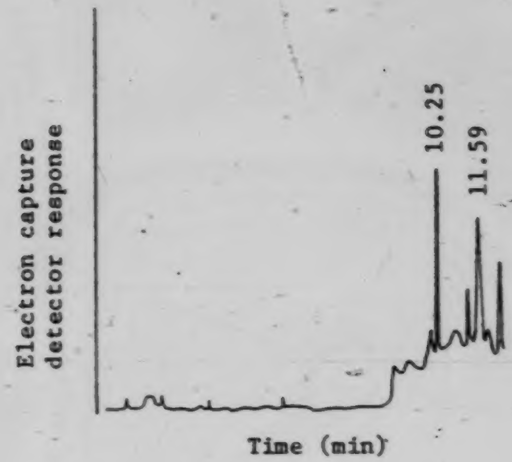


Figure 17. The GC trace of an extract of blood (300 ul) treated with acetic anhydride and pentafluoropropionic anhydride. No phenelzine or internal standard was added to the sample prior to derivatization and analysis.

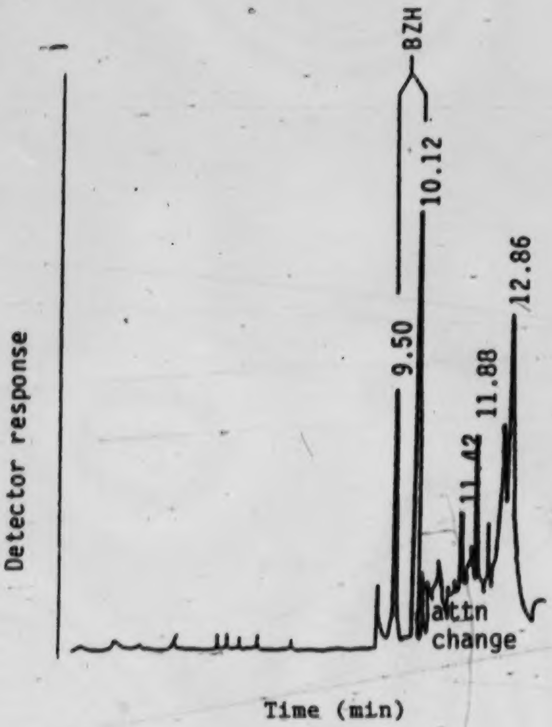


Figure 18. The GC trace of a solvent extract of a blank bile sample (20 ul) treated with acetic anhydride and pentafluoropropionic anhydride. The internal standard benzylhydrazine (3.0 ug/sample) was added prior to analysis. The retention time of the derivatives of benzylhydrazine are 9.50 min and 10.12 min. The conditions used for the GC separation of the components in the derivatized extract are given in the text.
BZH = acetic anhydride/PFPA derivatized benzylhydrazine.

3.1.2 Analysis of Phenylacetic acid

PAA is an *in vivo* metabolite of PLZ (98). This acid is also formed endogenously as a consequence of normal intermediary mammalian metabolism (193). Most methods currently available for the analysis of PAA lacked the sensitivity required to detect low levels of this acid in biological fluids. Diazomethane and trimethylsilyating reagents are available for the derivatization of PAA and its ring hydroxylated analogs (193,226,232,305). Although the GC characteristics of PAA are improved by this procedure, the products which are formed are insensitive to the ECD and NPD. In addition, trimethylsilylation of carboxylic acids is known to produce derivatives which are unstable (230). To form sensitive derivatives for the low level analysis of PAA, several other derivatizing reagents were developed. Lingemann et al. (195) converted PAA to a NPD-sensitive product by reacting it with a primary or secondary amine. The limit of detection of the PAA derivative was not given; however, the procedure was insensitive to concentrations of BA below 100 ng. Conversion of PAA to ECD-sensitive pentafluoropropyl and trifluoroethyl derivatives was successful (234,237,241,306) but low level detection of these derivatives required HRMS. In order to investigate the conversion of PLZ to PAA a new analytical procedure was required for the analysis of the metabolite. A sensitive GC procedure was envisaged which would allow identification of the derivatized acid by GC/MS. Once developed, this analytical procedure was used to study the formation of PAA from PLZ, in an isolated rat liver perfusion study.

The GC analysis of PAA was investigated following its derivatization with PFBA, an esterifying reagent not previously utilized for the GC analysis of PAA. This reagent readily reacted with PAA, yielding a derivative which was extremely sensitive to ECD. As little as 6 pg of this derivative could be detected by GC/ECD. Other urinary organic acids BA, p-OHPAA and m-OHPAA, could also be derivatized with PFBA, following their isolation from urine. PFBA derivatized BA, PAA, p-OHPAA and m-OHPAA were identified by their EI/MS fragmentation patterns (FIG 19-21).

Several inorganic and organic acids were evaluated as catalysts for the esterification reaction (Table 2). Of those investigated, only trifluoroacetic acid effectively catalyzed the esterification of PAA with PFBA. Optimal conditions for the derivatization of PAA with PFBA were obtained by heating the reaction mixture for 2 h at 80°C, in the presence of a 10 fold excess of PFBA in trifluoroacetic acid. When HCl in diethyl ether was used as a catalyst, the derivative which formed possessed a GC retention time (6.88 min) almost identical to PFBA-derivatized PAA (6.84). However, mass spectral and GC analysis of this derivative indicated that the product formed was likely the result of diethyl ether reacting with PFBA, forming pentafluorobenzylethyl ether (Fig 22).

Calibration graphs were constructed by carrying known quantities of PAA (0-3.5 ug) and 30 ul of the internal standard solution (I.S.; 1-naphthoic acid 1.4 mg/100 ml) through the derivatization procedure. Standard curves generated by plotting each GC peak area ratio (peak area of derivatized PAA/ peak area of derivatized I.S.) versus the quantity of PAA, were linear over the concentration range tested (Fig 23). Similarly prepared standard curves for BA were also linear over the same concentration range (0-2.8 ug/ml) (Fig 24).

To further evaluate this analytical procedure, total PAA and BA were quantified in urine, saliva and blood. Both urine and saliva were collected from healthy adult subjects. The blood sample was obtained from male Sprague-Dawley rats (400 g) by the heart puncture method. Levels of BA and PAA isolated from urine are given in table 3. The values of total PAA and BA in human urine agree favorably with those found in the literature (192,232). The quantity of PAA and BA detected in human saliva was 0.64 and 0.13 ug/ml, respectively. The level of PAA and BA was also determined in rat blood. The quantities detected were 0.1 ug/ml and 0.56 ug/ml, respectively. Once isolated from these fluids (urine, saliva or blood), PAA derivatized with PFBA could be easily separated by GC from other constituents present in the extract. Typical GC traces of PFBA treated extracts of urine before and after acid hydrolysis of conjugates are depicted in figures 25 and 26. The urine volumes extracted before and after acid hydrolysis were 1 ml and 0.05 ml, respectively. PFBA derivatized extracts of unhydrolyzed

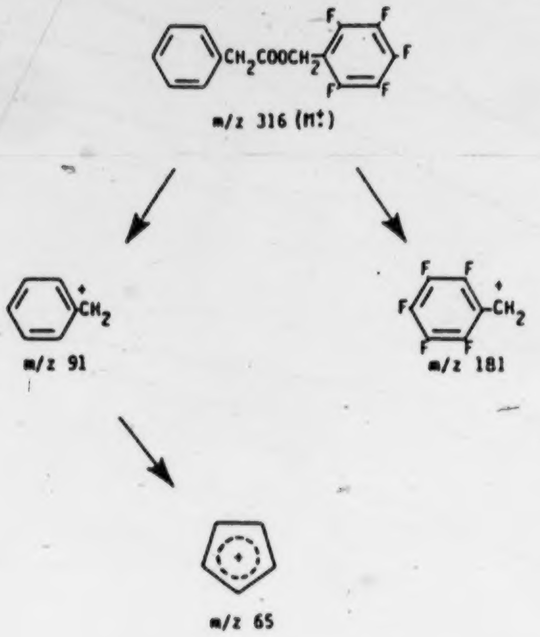
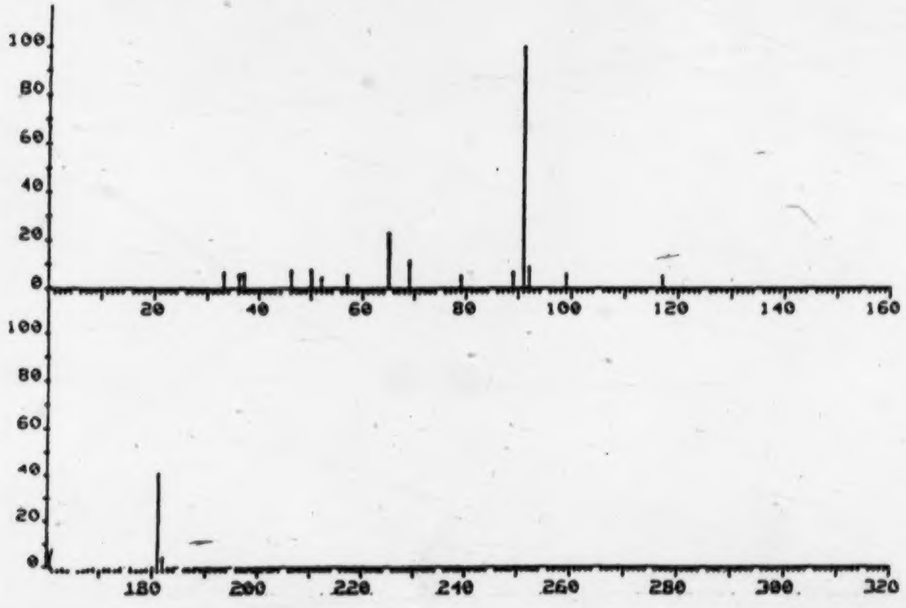


Figure 19. The proposed electron impact fragmentation pattern of phenylacetic acid esterified with pentafluorobenzyl alcohol. Only those ions which are diagnostic are represented. The actual electron impact mass spectrum of PFBA-derivatized phenylacetic acid is depicted below.



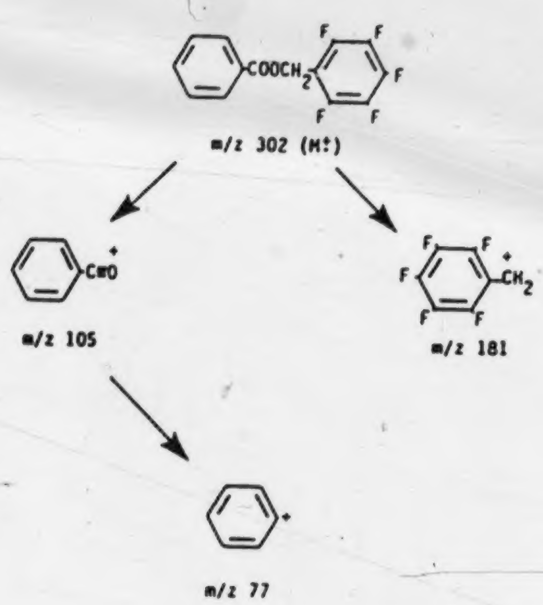
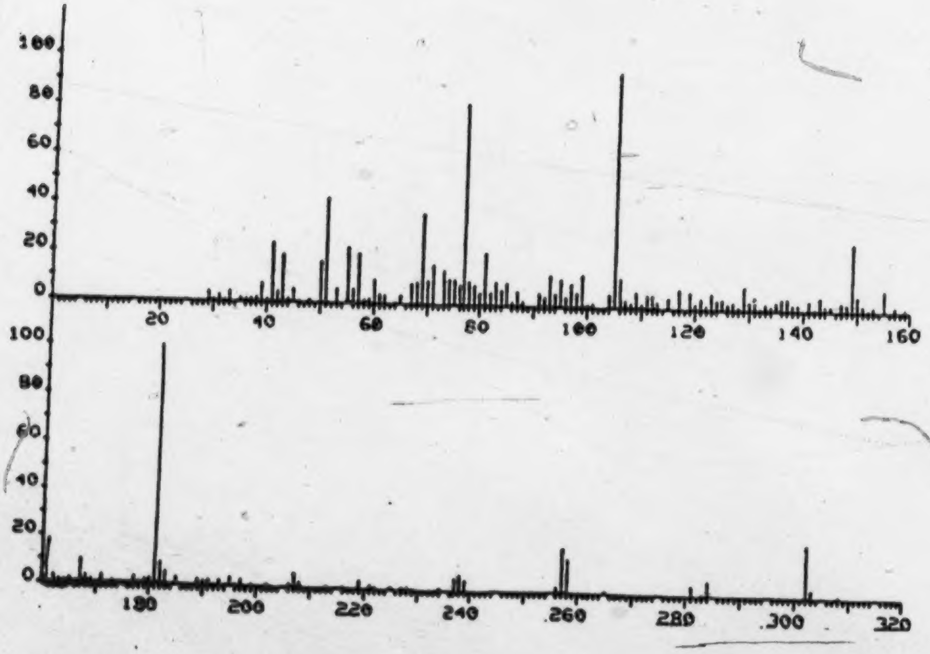


Figure 20. The proposed electron impact fragmentation pattern of benzoic acid esterified with pentafluorobenzyl alcohol. Only those ions considered diagnostic are represented. The actual electron impact mass spectrum is depicted below.



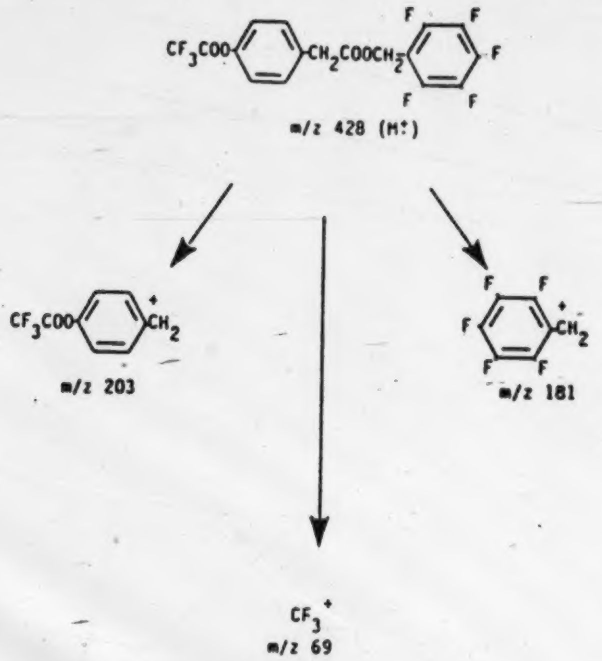


Figure 21. The proposed electron impact fragmentation pattern of p-hydroxyphenylacetic acid esterified with pentafluorobenzyl alcohol. Only those ions considered diagnostic are represented. The actual electron impact mass spectrum is given below. The fragmentation pattern of m-hydroxyphenylacetic acid, similarly treated, is identical to the fragmentation pattern of the p-isomer.

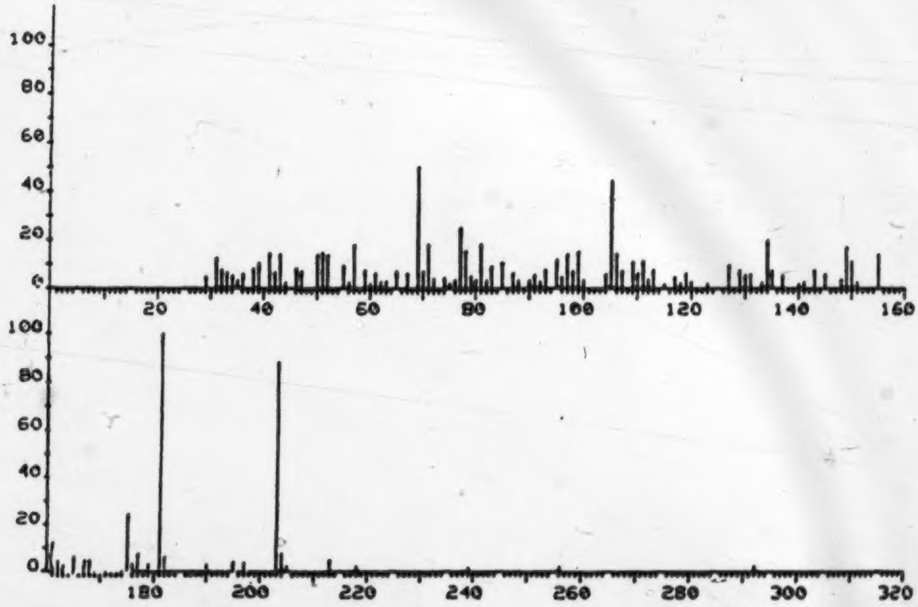


Table 2. Acid catalysts employed in the esterification of phenylacetic acid with pentafluorobenzyl alcohol. All the reactions were carried out at 80°C for 2 h.

Acid catalyst
1,4-dioxane/HCl
diethyl ether/HCl
conc. HCl
conc. H_2SO_4
trifluoroacetic acid

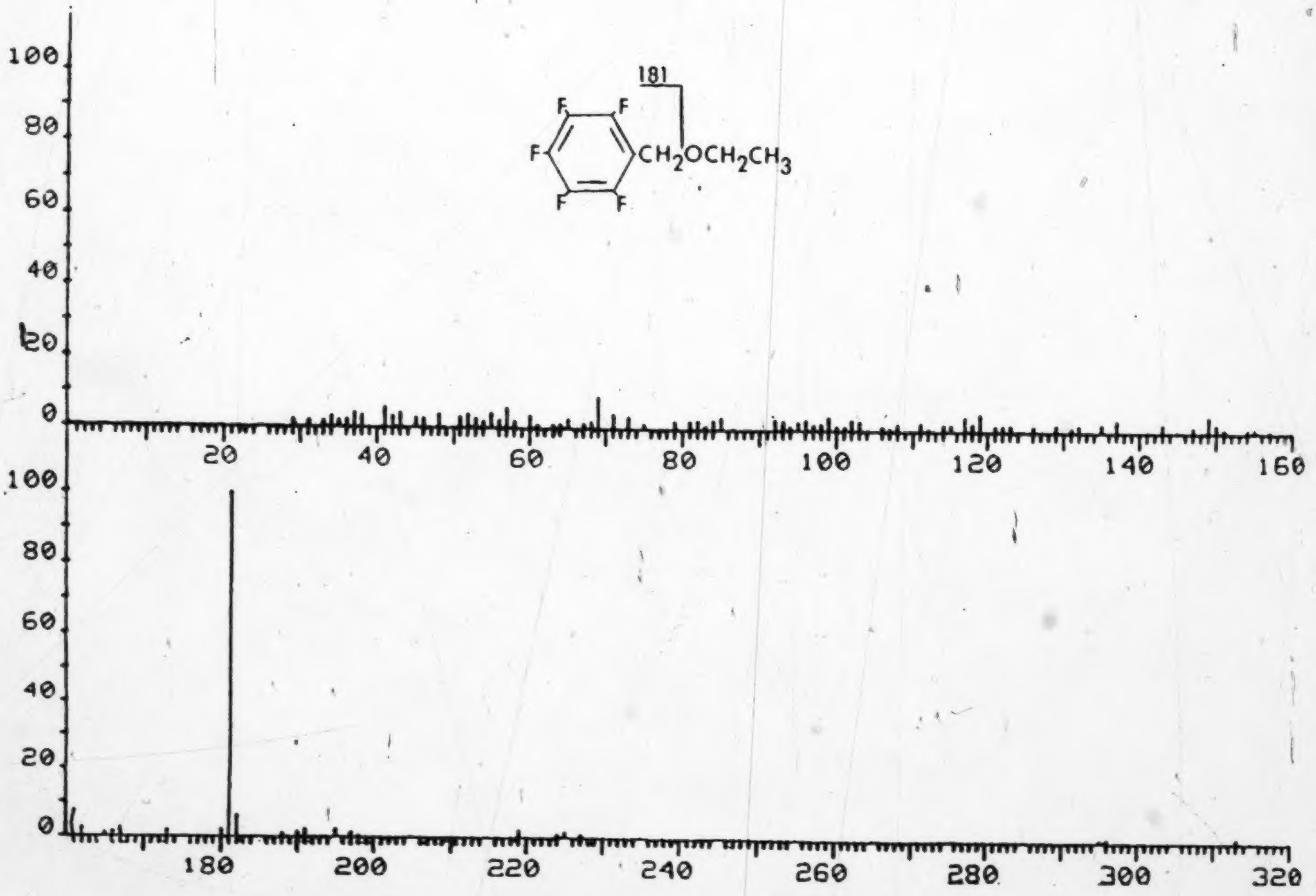


Figure 22. The electron-impact mass spectrum of the product formed when diethyl ether/HCl was reacted with pentafluorobenzyl alcohol. The identity of the major fragmentation ions are depicted above.

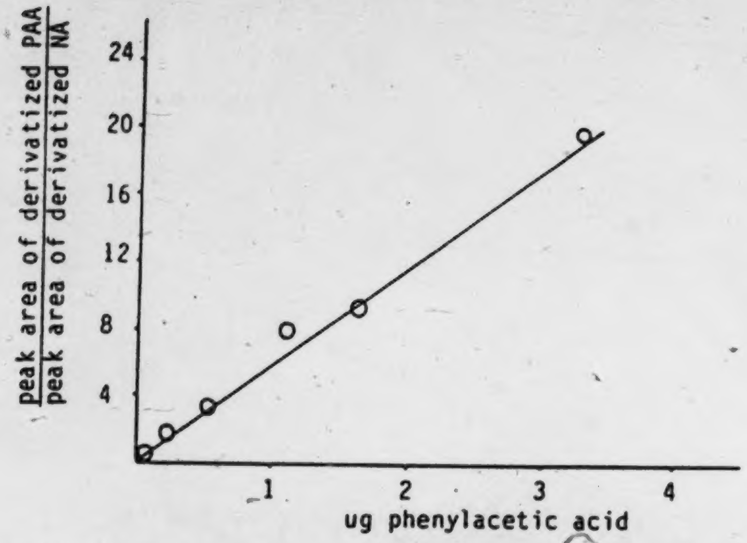


Figure 23. The calibration graph of the pentafluorobenzyl ester of phenylacetic acid in the concentration range 0-3.5 ug/sample. GC conditions are given in the text. 1-Naphthoic acid (NA) was used as internal standard.

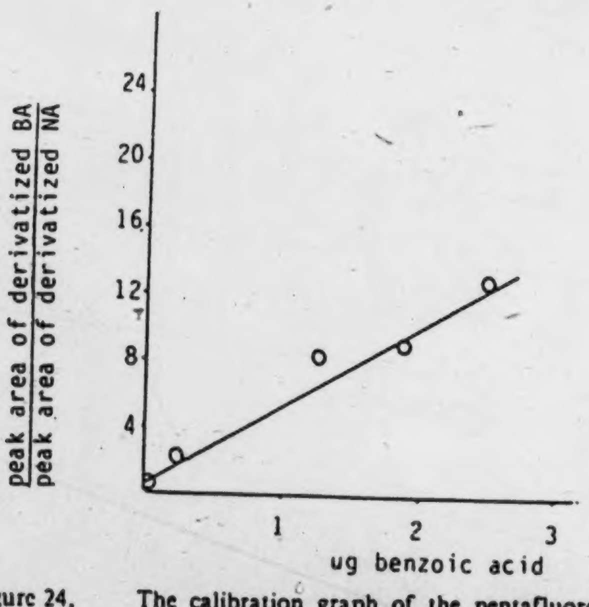


Figure 24. The calibration graph of the pentafluorobenzyl ester of benzoic acid (0-2.5 ug/sample). The GC conditions used are given in the text. 1-Naphthoic acid (NA) was used as the internal standard.

urine only contained BA, m-OHPAA and p-OHPAA, whereas PFBA derivatized extracts of urine after acid hydrolysis contained these three compounds in addition to PAA. Since urinary BA is known to be present in a conjugated form, as the glycine conjugate (hippuric acid) (130), it was deduced that during the derivatization procedure the amide bond of hippuric acid was hydrolyzed and the freed BA subsequently derivatized with PFBA. This hypothesis was confirmed by subjecting authentic hippuric acid to the same procedure. The m-OHPAA and p-OHPAA were isolated from urine, prior to hot acid treatment. This suggests that these two acids are present in the free form or as labile amino acid conjugates, which are hydrolyzed during derivatization. It is unlikely that glucuronide conjugates of these acids would be isolated from the urine sample using a solvent extraction procedure, as was done in this study.

It was observed that clean-up of blood or saliva samples, by solvent extraction at pH 9 significantly reduced the quantity of BA and PAA which could be recovered. Significant quantities of these acids are believed to be lost during extraction from basic samples into dichloromethane.

To demonstrate the usefulness of PFBA as a routine derivatizing reagent for the GC analysis of organic acids, a number of organic and amino acids (ibuprofen, 1-phenylcyclopentanoic acid, m- and p-aminobenzoic acid, 1-naphthoic acid, alanine, phenylalanine, and α-methyltyrosine) were derivatized with this reagent. Standard samples, which consisted of 1 ug of each organic acid dissolved in 2 ml deionized water, were isolated by solvent extraction and derivatized with PFBA. The amino acids were isolated from the aqueous sample by evaporating the sample to dryness. The structures of the derivatized acids were identified by their EI fragmentation patterns (Fig 27-33).

All the PFBA-derivatized acids had extremely good responses to ECD. With some of the amino and phenolic carboxylic acids, simultaneous derivatization of all the functional groups present could be obtained by adding a small amount of TFAA to the reaction mixture (PFBA/trifluoroacetic acid).

Table 3. The quantities of phenylacetic acid and benzoic acid isolated from urine of healthy human subjects. The urine was collected over a 24 h period. The values obtained represent total phenylacetic acid and total benzoic acid present (i.e. free + conjugated).

	mg/24 h urine (Mean ± S.D.)
PAA	93.1 ± 33.5
BA	210.8 ± 66.7

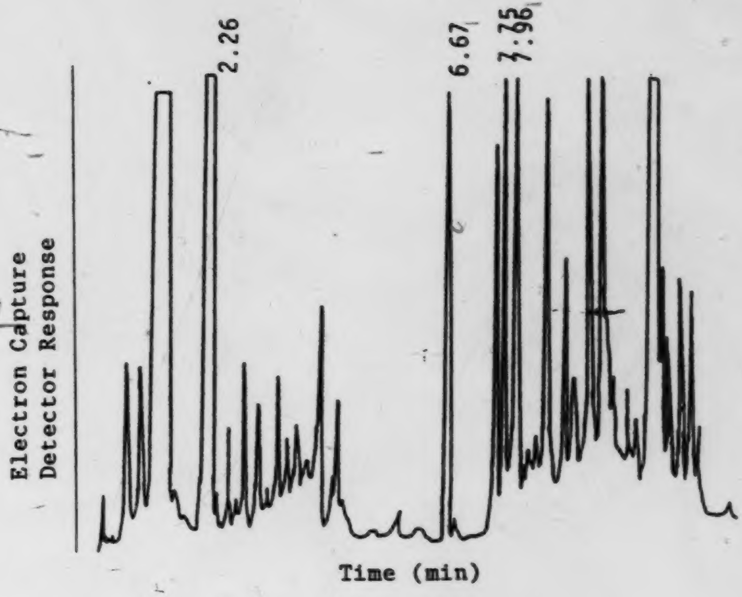


Figure 25. The GC trace of an extract of unhydrolyzed urine (1 ml) derivatized with pentafluorobenzyl alcohol and trifluoroacetic anhydride. The GC peaks are identified by their retention times: pentafluorobenzyl alcohol (PFBA) (2.26 min), PFBA-derivatized benzoic aicd (6.67 min), PFBA-derivatized m-hydroxyphenylacetic acid 7.75 min), and PFBA-derivatized p-hydroxyphenylacetic acid (7.76 min). The remaining GC peaks were not identified.

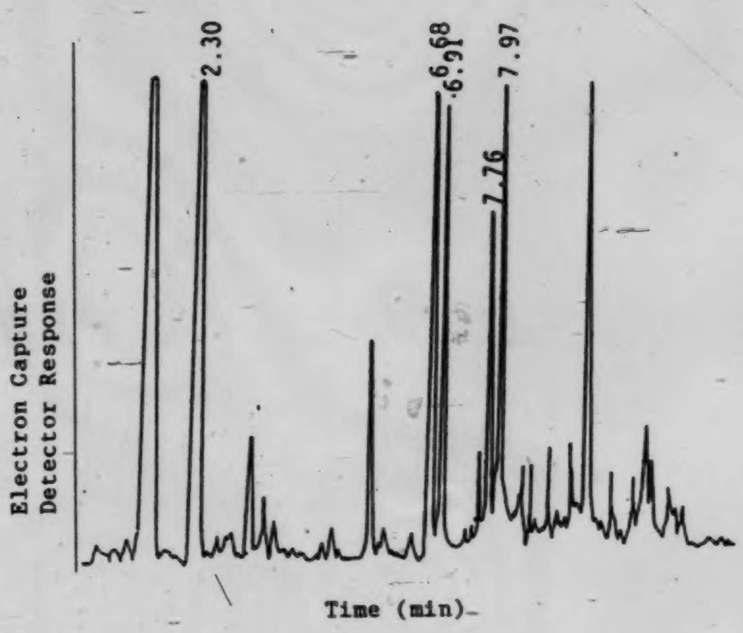


Figure 26. The GC trace of an extract of hydrolyzed urine (0.05 ml) derivatized with pentafluorobenzyl alcohol (PFBA) and trifluoroacetic anhydride. The peaks in the trace are identified by their retention times: pentafluorobenzyl alcohol (2.30 min), PFBA-derivatized benzoic acid (6.68 min), PFBA-derivatized phenylacetic acid (6.91 min), PFBA-derivatized m-hydroxyphenylacetic acid (7.76 min) and PFBA-derivatized p-hydroxyphenylacetic acid (7.97 min). The remaining GC peaks were not identified.

The amino acids (alanine, phenylalanine and α-methyltyrosine) were derivatized with the PFBA/TFAA/trifluoroacetic acid derivatizing solution. GC analysis of derivatized α-methyltyrosine indicated that two products had been formed during the derivatization procedure. One product was identified as the expected PFBA/TFAA-derivatized α-methyltyrosine, while the second appeared to be a decomposition product. The mass spectrum of the decomposition product confirmed that this product was derived from PFBA/TFAA-derivatized α-methyltyrosine. In particular, the fragment ion m/z 203 ($CF_3COOC_6H_4CH_2+$) was observed in the mass spectrum of the decomposition product. The identity of this compound could not be determined from its EI fragmentation pattern. The poor sensitivity observed with phenylalanine, compared to the other derivatized acids, suggests this compound either is insensitive to the detector or was also partially decomposed during derivatization. No decomposition product was detected during the GC analysis of PFBA/TFAA-derivatized phenylalanine. Low levels of alanine (3 ng on-column) could be easily detected by GC/ECD, following its derivatization with PFBA/TFAA. This amino acid appeared to be stable to the derivatization procedure.

It is evident that this reagent can be utilized to derivatize organic acids (carboxylic acids). In the present study this reagent was used for the analysis of PAA in the perfusate obtained by infusing an isolated rat liver with PLZ and to determine normal levels of PAA in urine of humans. Analysis of PAA in the urine of rats administered PLZ, was not done, however. In mammals, large quantities of PAA are derived from several sources other than PLZ. It would be difficult to demonstrate changes in the level of PAA caused by chronic PLZ administration. One possible method which could be used to determine the PAA derived from PLZ would be to use PLZ labelled with deuterium atoms. In this manner only the PAA produced from PLZ could be detected.

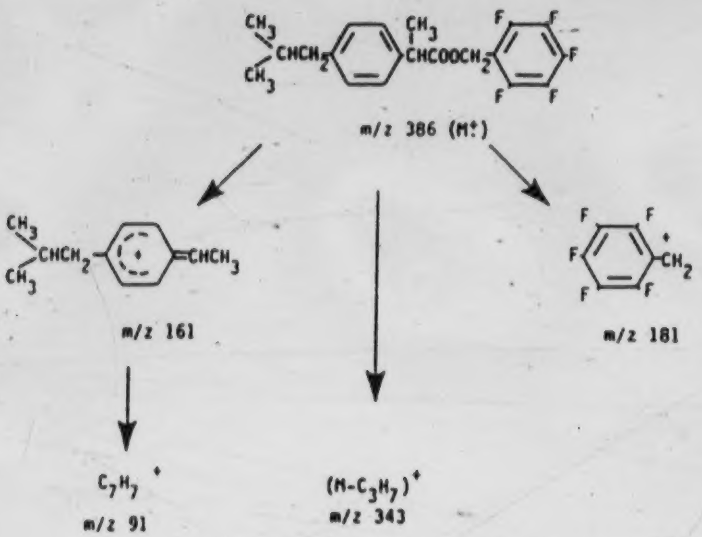
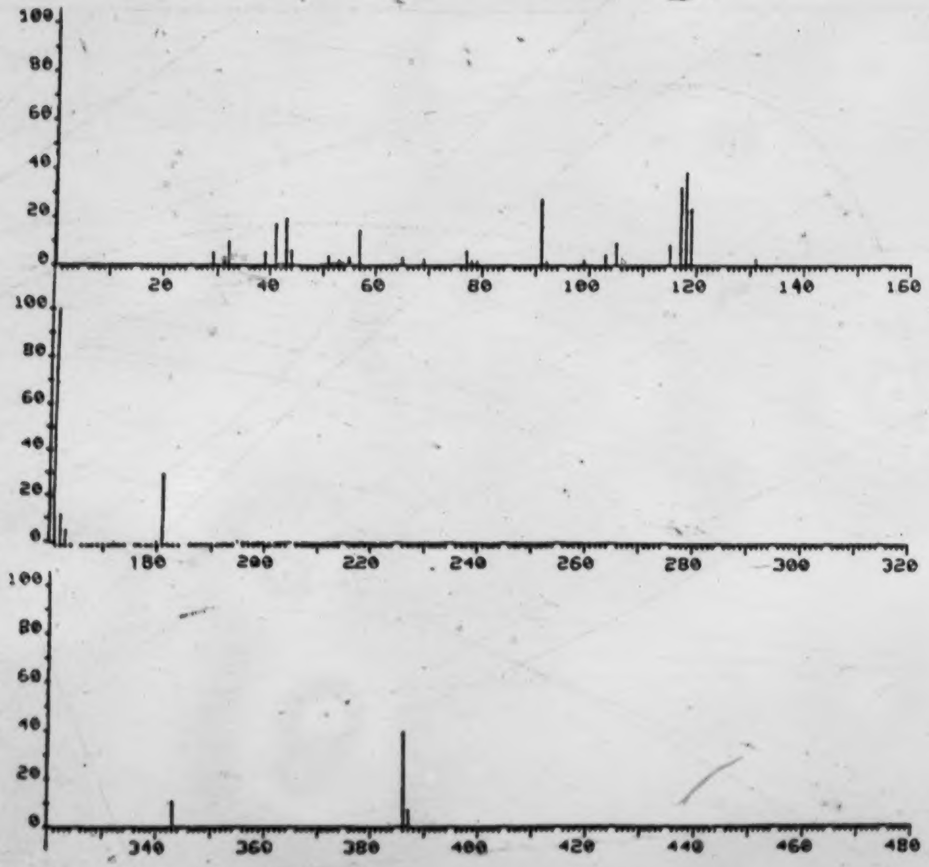


Figure 27. The proposed electron impact fragmentation pattern of ibuprofen esterified with pentafluorobenzyl alcohol. Only those fragment ions considered diagnostic are represented. The actual mass spectrum is given below.



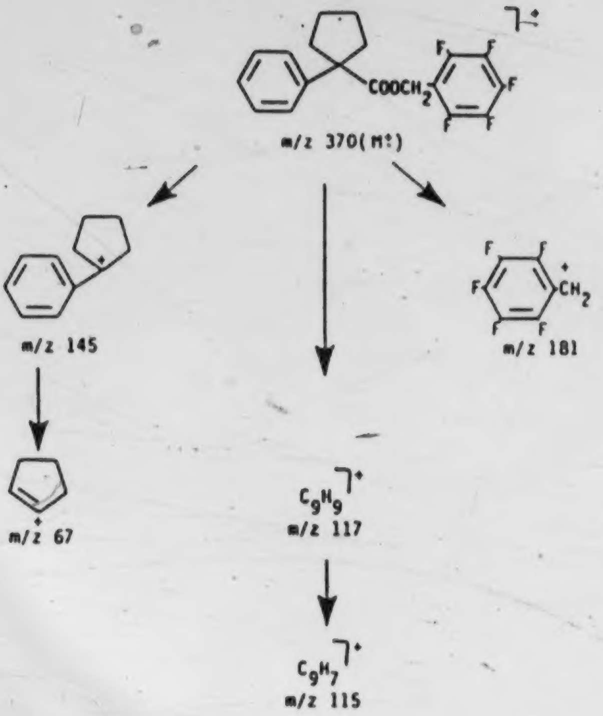
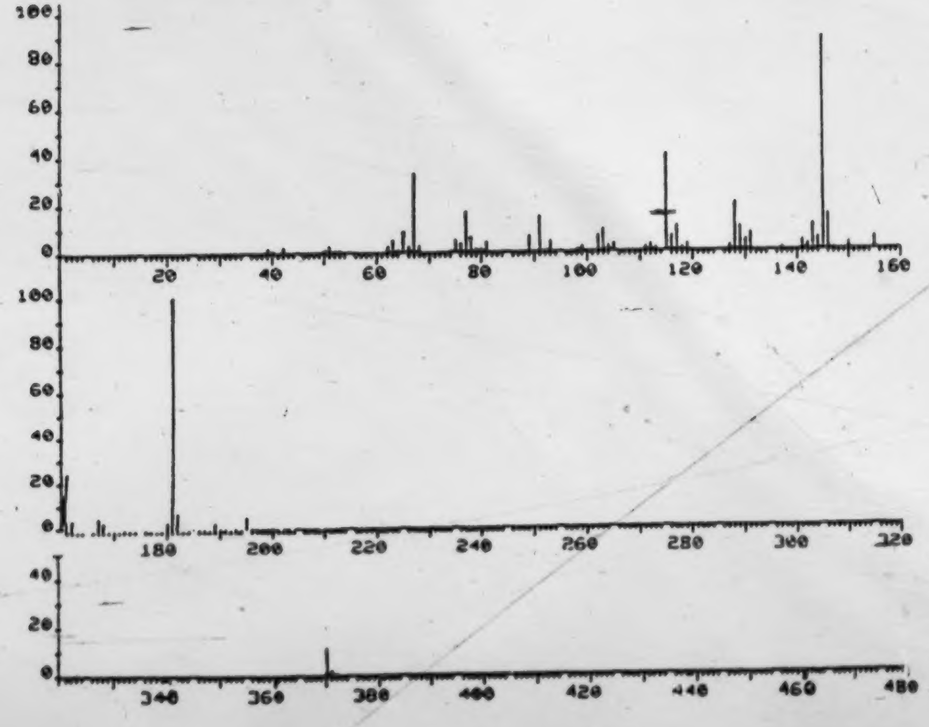


Figure 28. The proposed electron impact fragmentation pattern of 1-phenylcyclopentanoic acid esterified with pentafluorobenzyl alcohol. Only those fragment ions considered diagnostic are represented. The actual electron impact mass spectrum is depicted below.



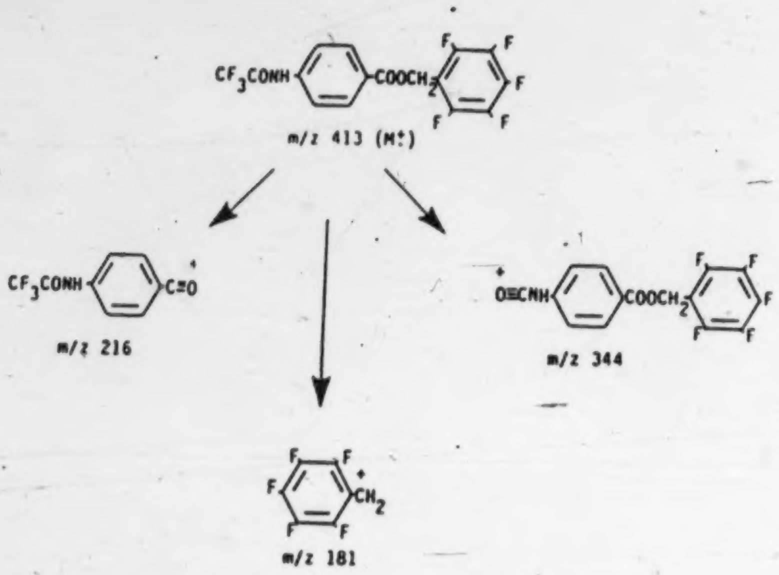
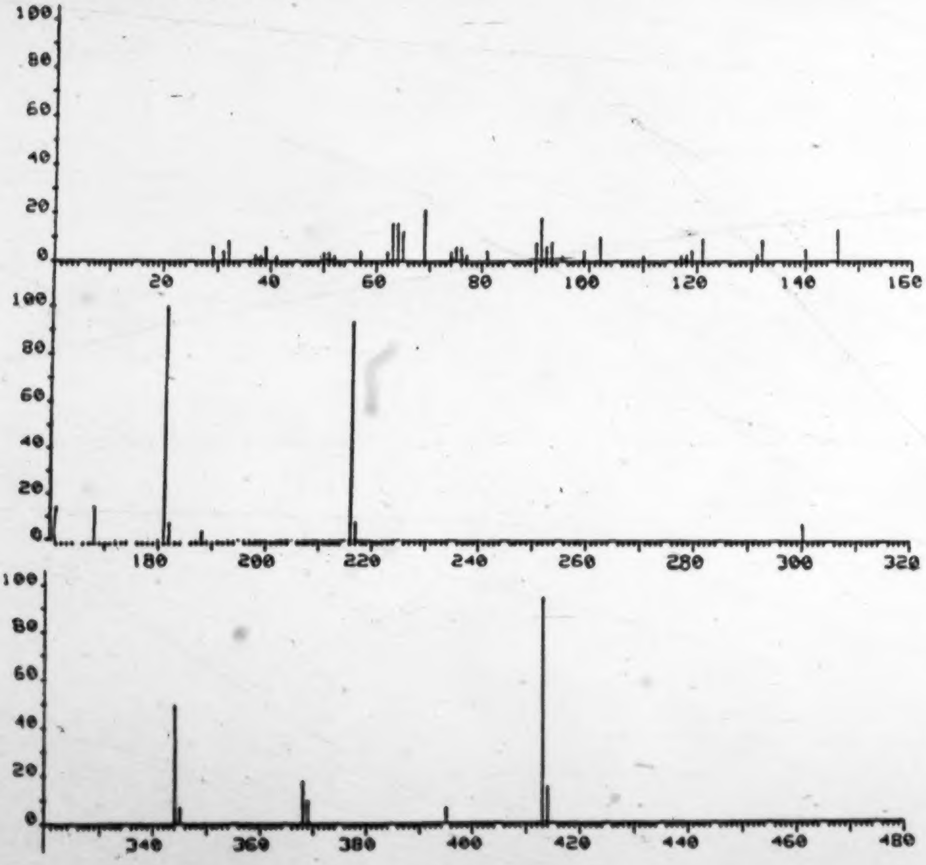


Figure 29. The proposed electron impact fragmentation pattern of p-aminobenzoic acid derivatized with pentafluorobenzyl alcohol and trifluoroacetic anhydride. Only those fragment ions considered diagnostic are represented. The actual mass spectrum is given below.



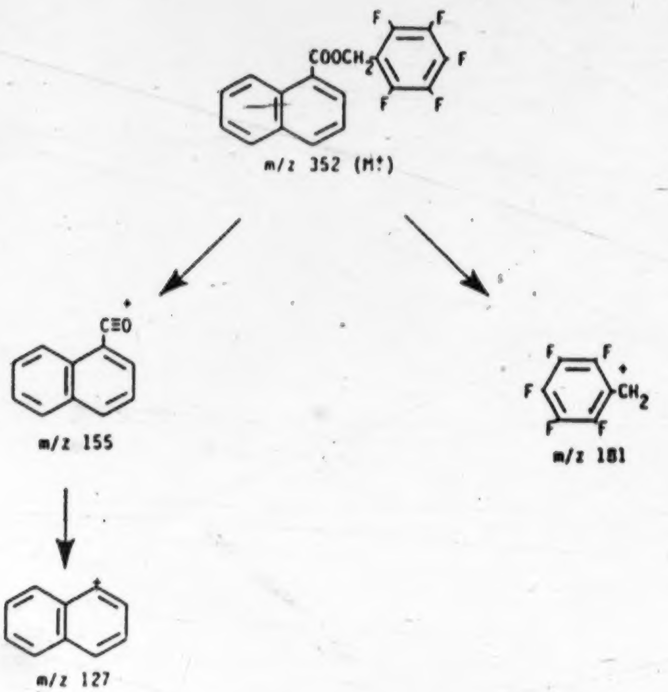
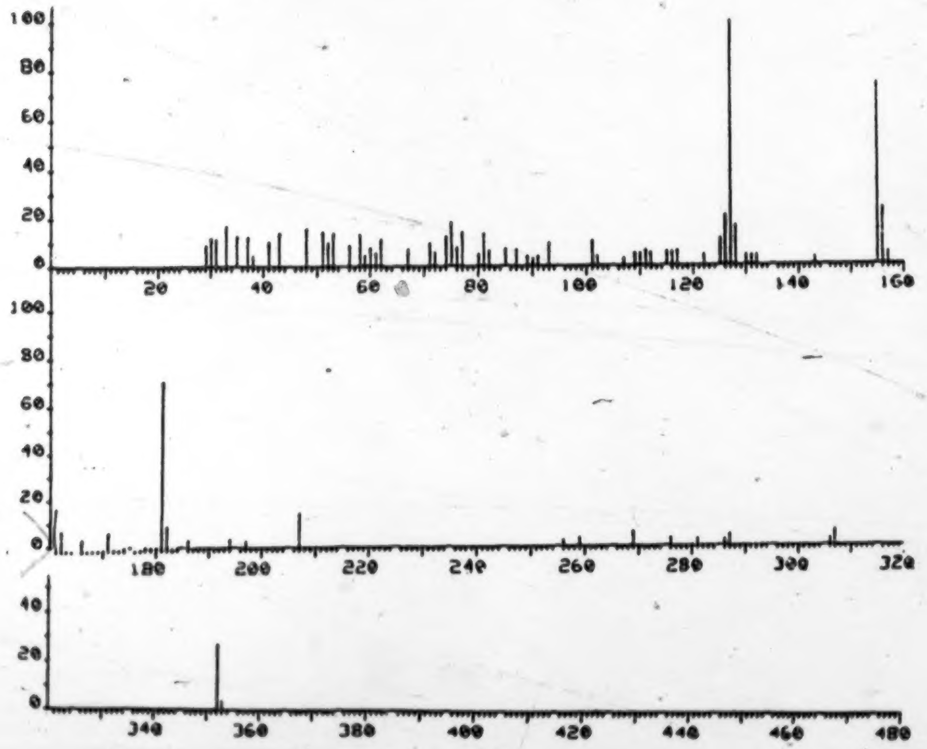


Figure 30. The proposed electron impact fragmentation pattern of 1-naphthoic acid esterified with pentafluorobenzyl alcohol. Only those fragment ions considered diagnostic are represented. The actual mass spectrum is depicted below.



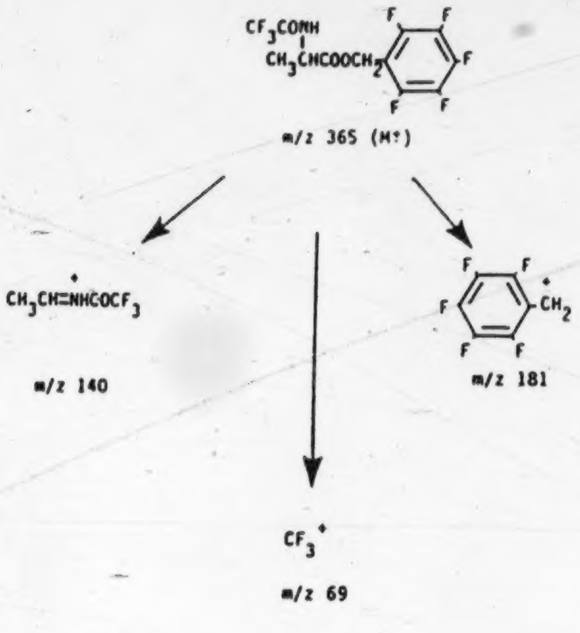
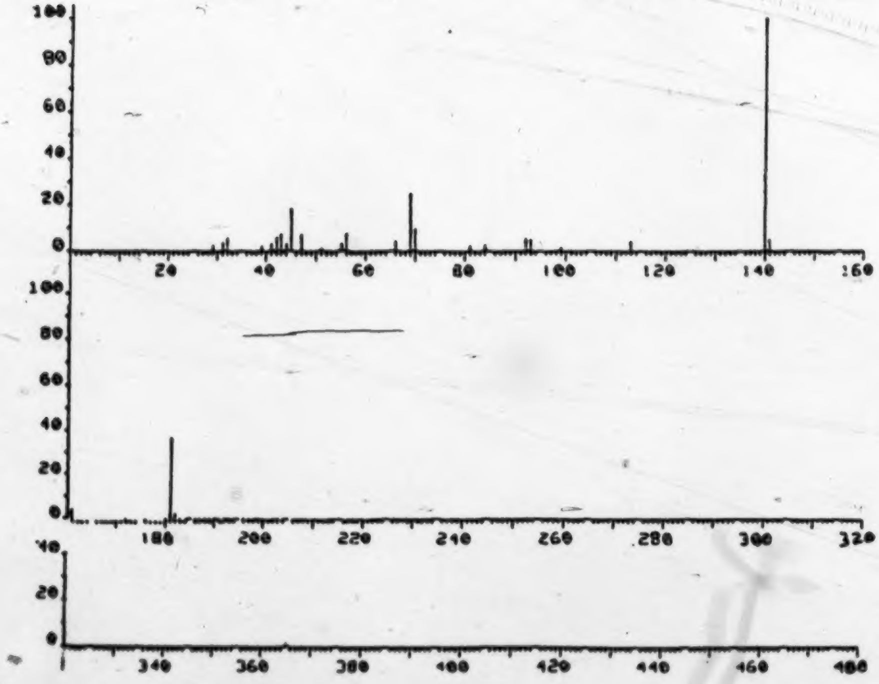


Figure 31. The proposed electron impact fragmentation pattern of D-alanine derivatized with pentafluorobenzyl alcohol and trifluoroacetic anhydride. Only those fragment ions considered diagnostic are represented. The actual mass spectrum is given below.



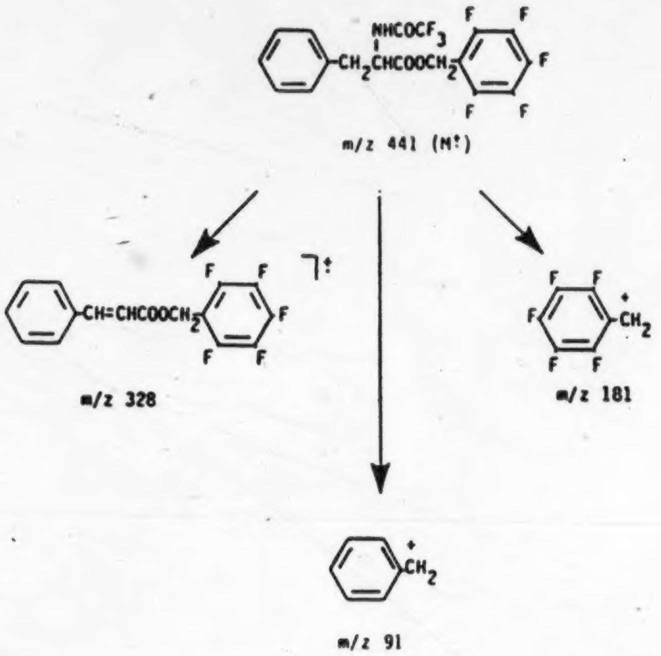
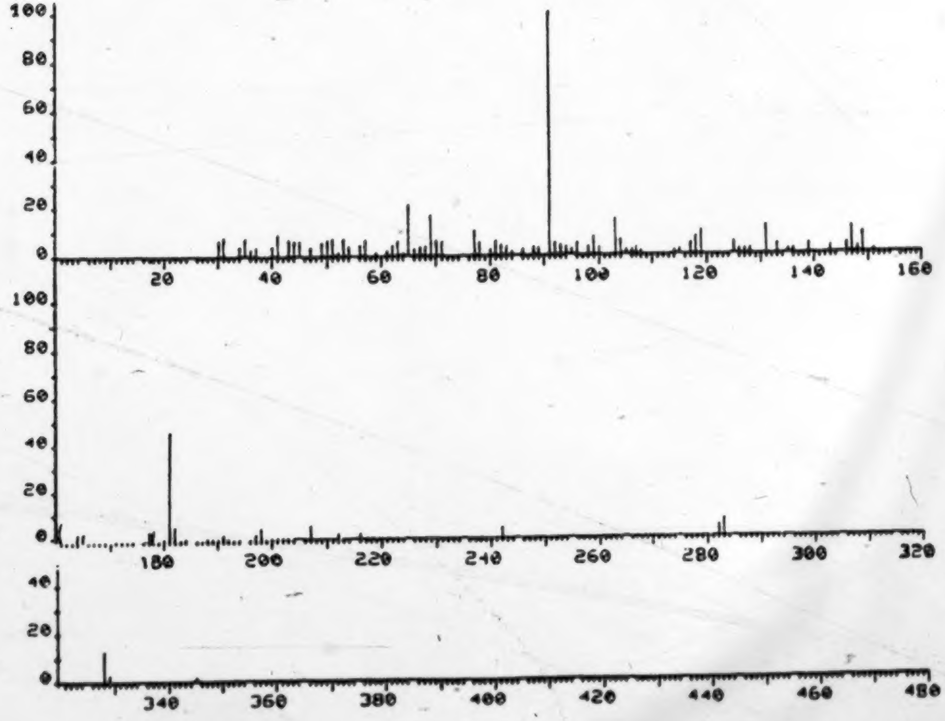


Figure 32. The proposed electron impact fragmentation pattern of D,L-phenylalanine derivatized with pentafluorobenzyl alcohol and trifluoroacetic anhydride. Only those fragment ions considered diagnostic are represented. The actual mass spectrum is given below.



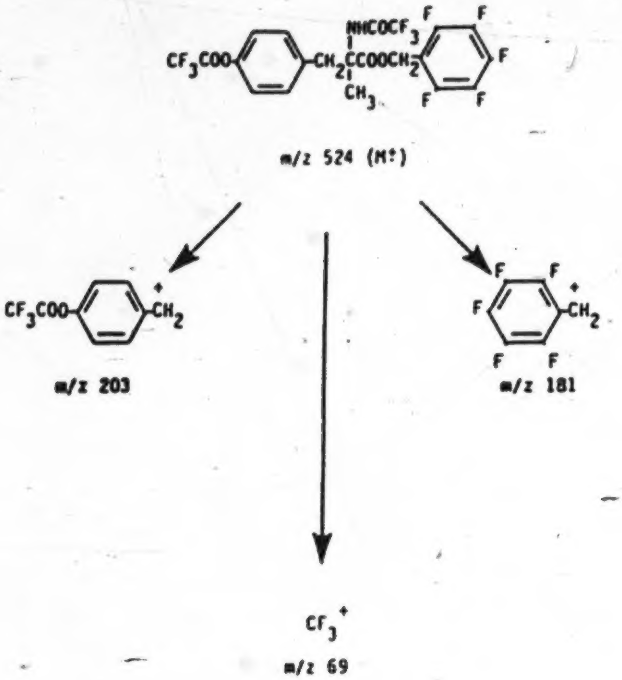
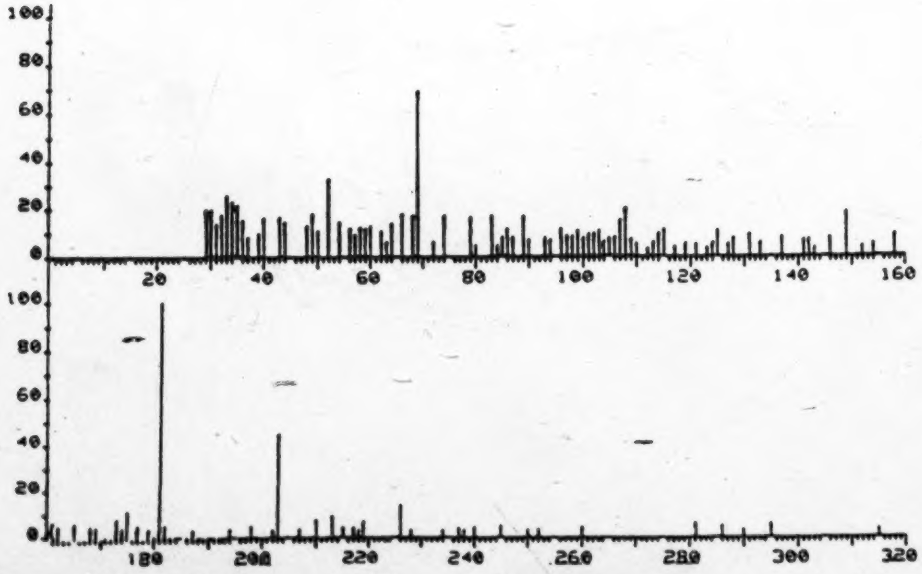


Figure 33. The proposed electron impact fragmentation pattern of α-methyltyrosine derivatized with pentafluorobenzyl alcohol and trifluoroacetic anhydride. Only those fragment ions considered diagnostic are represented. The actual mass spectrum is given below.



3.1.3 GC Analysis of Neutral Metabolites

In a previous study, using an *in vitro* microsomal preparation, it was demonstrated that EBZ could be formed from PLZ (13). In the present investigation a volatile metabolite, other than carbon dioxide, was observed in the expired air of rats dosed with PLZ. To identify this unknown volatile compound, a procedure for its isolation from expired air was required.

3.1.3.1 General Considerations

Several techniques, using either solid adsorbants (197,307,308,309) or cryogenic liquid traps (107,197), have been devised for the isolation of volatile compounds from enviromental or expired air samples. The system selected often depends on the type of compound being isolated, the volume of air being sampled and the sampling rate to be used (310). Optimally the system should be capable of efficiently trapping the volatile compound at a high flow (sampling) rate. Sampling efficiency of the trapping system is not only dependent on the flow (sampling) rate but also can be affected by the concentration of the compound in the air sample (310). For example, a number of methods used for the isolation of volatile compounds have a minimum concentration below which the sampling efficiency is reduced to zero (310).

The trapping of volatile compounds from the expired air of animals poses several additional problems, commonly not encountered with environmental samples. Since the animals are normally housed in metabolism cages, the flow rates used to sample the air must be rapid, to ensure proper aeration of the animal. Frequently the volatile metabolite is expired over long periods of time. The procedure used to isolate the expired volatile compound must be suitable for prolonged collection periods. Often the sampling efficiency of a technique is significantly reduced after a certain period of time (197). The method used must permit sample concentration, once the volatile compounds have been isolated, for subsequent GC/MS analysis. When using a solid adsorbant this can be achieved by either solvent desorption or, in the case of Tenax GC®, by thermal desorption. In the latter procedure the sample is eluted by heat into a stream of gas and carried directly onto the

GC column. If a cryogenic solvent trap is used, the solvent should be sufficiently volatile so that it can be evaporated to a small volume, under a stream of nitrogen, to concentrate the sample. It has been suggested, however, that solvent evaporation can result in some loss of sample (197).

3.1.3.2 Systems used to Trap Volatiles

Common adsorbants used for the isolation of various classes of compounds (pesticides, alcohols and hydrocarbons) include activated charcoal, XAD, Porapak, Chromosorb and Tenax GC (197). The most popular of these adsorbants appears to be Tenax GC, because of its thermal stability (197). This adsorbant was used successfully to isolate toluene from an air sample expired by human subjects exposed to this solvent (198). Cryogenic traps have also been used effectively to trap volatile compounds from air samples. Propane was isolated from the expired air of rats given iproniazid using this method (107). Trapping efficiency of this procedure was 70%.

New approaches to the isolation of volatile compounds have been described. Krotoszynski et al. (311) described a trapping procedure which initially involved the collection of the expired air from human subjects into a teflon bag. The contents of the bag was then passed through a Tenax GC® adsorption column to trap the volatiles present. By using this procedure, the rate at which the air sample was passed through the adsorbant could be easily controlled. This technique, however, is not suitable for the trapping of volatiles expired by animals. Animals cannot be trained to expire into a teflon bag.

In the current study, both solid adsorbants and cryogenic solvent traps were evaluated (Table 4). The expired volatile compound, believed to be EBZ, could only be effectively trapped in pentane (100 ml) cooled to -32°C in a dry ice acetone bath. Pentane was selected as the trapping solvent because of the miscibility of EBZ in this solvent and the fact that the boiling point of pentane is much lower than that of EBZ. The solvent could be easily evaporated without significant loss of the trapped EBZ. In addition, the method proved effective for the isolation of EBZ over prolonged collection periods. In this study it was confirmed that little of the trapped EBZ was lost over the prolonged collection period.

The three solid adsorbants tested were either of little use or ineffective in trapping EBZ from air samples, presumably as a result of the rapid flow rate that had to be used. In previous studies it was determined that optimal flow rates when solid adsorbants were employed to isolate pesticides from enviromental air samples ranged from 3-9 ml/min (197). A flow rate of 800 ml/min had to be used in the current study. An XAD-2 polystyrene resin could not be used for the isolation of EBZ because it was found to be contaminated with a large quantity of EBZ, which could not be removed prior to its use. Even after extensive organic solvent washing, sufficient EBZ remained in the resin to interfere with the trace analysis of this compound. Tenax GC, although used previously for the isolation of hydrocarbons from expired air (198), was not tested in this study. With Tenax GC, the trapped volatiles are normally eluted thermally from the adsorption column then injected directly onto the GC column. The technique of on-column injection was unavailable for this study. Although other adsorbants are available, they were not utilized in this study. It was believed that they too would be ineffective since the required sampling flow rate was so rapid.

3.1.3.3 Quantitation of Trapped Volatiles

Few methods have been developed for the quantitation of volatile compounds isolated from air samples. The procedure most frequently used involves adding a standard quantity of compound to the solid adsorbant and then drawing air through the adsorbant for a predetermined length of time. The efficiency of the isolation procedure can then be determined by measuring the quantity of the compound which was retained on the adsorbant. Another method used involved the passing of a known quantity of compound, preferably radiolabelled, through the solid adsorbant or cryogenic solvent. The trapping efficiency can be determined by estimating the quantity of compound recovered from the air sample. The major drawback to both procedures is that neither simulates what occurs in

Table 4. Adsorbants tested for their ability to trap ethylbenzene from expired air. The solid adsorbants were suspended in the solvent using a magnetic stirrer.

Adsorbant	Solvent	Volume
activated charcoal (3-5 g)	n-decane	30 ml
	water	50 ml
	n-pentane	50 ml
XAD-2 resin (3 g)	n-pentane	50 ml
	dry*	
Chromosorb W	dry*	-

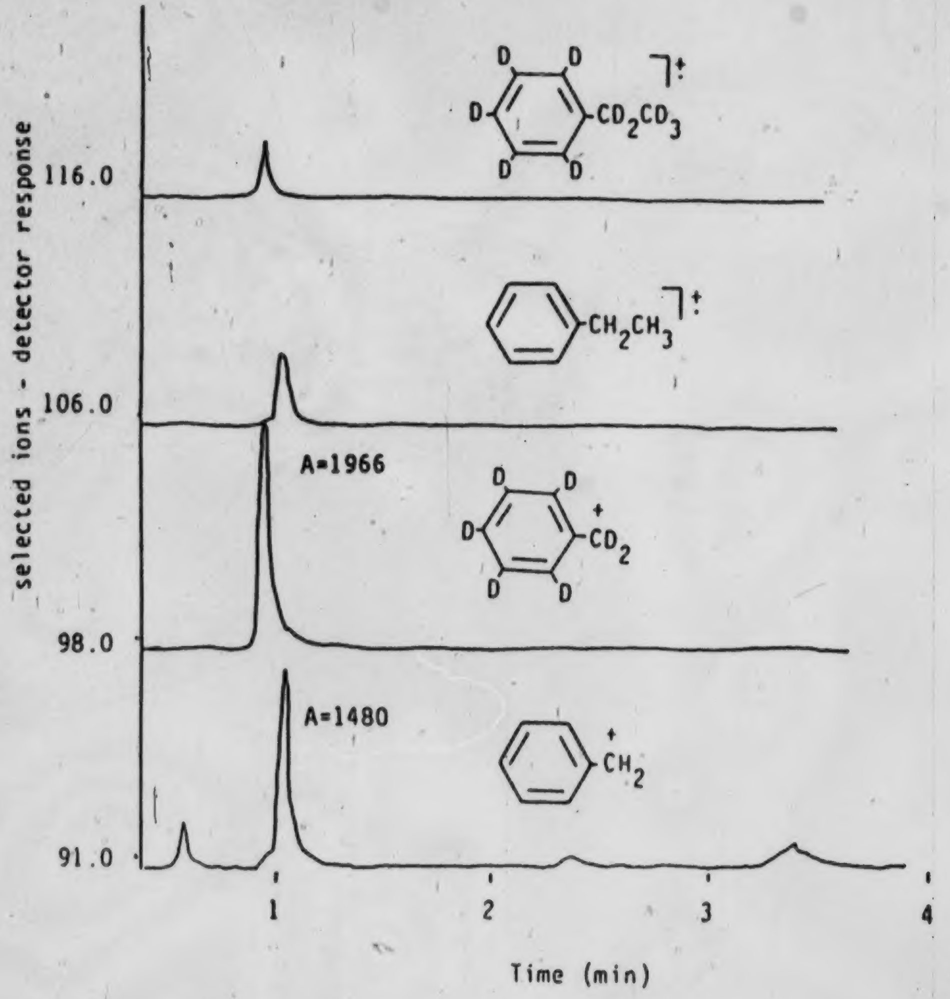
* the solid adsorbant was packed tightly into a 3 mm i.d. glass GC column.

an actual experimental situation. During experiments with animals, compounds are expired slowly over a long period of time.

In an attempt to overcome the limitations of previously used methods of quantitation, an internal standardization method was evaluated. In the procedure, D_{10}-EBZ was utilized as an internal standard. Detection of the internal standard and EBZ was accomplished by GC/MS using selective ion monitoring (SIM). The ions chosen for detection were the molecular ions m/z 106, 116 and the fragment ions m/z 91, 98 for EBZ and D_{10}-EBZ, respectively (Fig 34). In this procedure, known quantities of EBZ and a standard amount of D_{10}-EBZ were carried through the isolation procedure and then analyzed by GC/MS-SIM. By dividing the peak area of the m/z 91 ion by the peak area of the m/z 98 ion and plotting the ratio obtained versus the quantity of EBZ, a calibration graph was constructed. However, the calibration graph obtained was nonlinear over the concentration range 0-150 ug of EBZ. This nonlinearity was attributed to a) changes in the flow rate caused by fluctuations in the strength of the in-house vacuum system and b) changes in the sampling efficiency as a result of differences in the concentration of EBZ volatized. Methods utilized to reduce the vaporization rate of the standard EBZ samples failed. By reducing the rate at which standard samples of EBZ are vaporized, the isolation of standard EBZ will more closely resemble what occurs during the actual experiment. The techniques tried included placing EBZ into a large volume of solvent (pentane) or by adsorbing EBZ onto a piece of cloth.

Since the sampling efficiency of the procedure used in this study was unknown, accurate quantitative estimation of the EBZ expired could not be determined. A standard quantity of D_{10}-EBZ was added to the trapping solvent prior to collection of the expired EBZ. The quantity of trapped EBZ was estimated by comparing the peak area of ion m/z 91 (EBZ) to the peak area of ion 98 (D_{10}-EBZ). This standard addition method only estimates the quantity of EBZ trapped. Only if the trapping efficiency of the procedure was known to be quantitative would the result obtained with this procedure represent the

Figure 34. Selective ion monitoring of ethylbenzene (ions 91,106) and the internal standard D_{10}-ethylbenzene (ions 98,116). Ethylbenzene was isolated from the expired air of rats dosed with phenelzine, by the method described in Materials and Methods. Quantitation of the ethylbenzene trapped was achieved by comparing the peak areas derived from D_{10}-ethylbenzene and those of ethylbenzene.



actual quantity of EBZ excreted in the expired air. Using this method 60 ug of the expired EBZ, from a rat treated with a single dose of PLZ, was trapped in the cryogenic solvent.

3.1.4 Metabolism and Pharmacokinetics of Phenelzine

Despite its frequent use in the treatment of atypical endogenous depression, little is known about the metabolism of PLZ. The observed low renal excretion of unaltered PLZ (1% of the dose) in man implies that metabolism or biliary excretion play an important roll in PLZ elimination. However, it would be unusual for a low molecular weight compound (PLZ; MW=134) to be eliminated by the biliary route unless it was conjugated prior to excretion. Besides the normal endogenous compounds eliminated in bile, the only other unconjugated compounds eliminated extensively by this route are quaternary ammonium organic compounds, such as procainamide etheobromide (291).

Clineschmidt and Horita (95) have demonstrated that PLZ is converted to PAA by the same enzyme (MAO) that this drug inhibits. Following a single intraperitoneal (i.p.) dose of PLZ to rats, a significant quantity of PAA (51% of the dose) was formed. These investigators demonstrated that PAA, derived from PLZ, could still be isolated in rats pretreated with tranylcypromine or pargyline, two MAO A and B inhibitors. During the dehydrazination of PLZ to form PAA, hydrazine is also formed (110). Only 3% of a dose of PLZ could be recovered as hydrazine in urine (332). Hydrazine itself is a potentially toxic metabolite since it is further metabolized by a free radical mechanism (347).

A second, controversial metabolite of PLZ is N-acetyl-PLZ. For many years the existence of this metabolite has been questioned (324). Evidence for the *in vivo* acetylation of PLZ is indirect; a) structurally-related hydrazine drugs (e.g. isoniazid) are known to be acetylated (313); or b) PLZ is converted to N-acetylphenezine *in vitro* by a highly purified rabbit liver N-acetyltransferase. Narasimhachari et al. (167) claim to have detected N-acetyl-PLZ in the urine of rats administered PLZ . In their study, both mono- and di-acetylated PLZ 15 and 16 were detected using GC/MS-SIM. The full EI mass spectrum of this compound isolated in rat urine was not recorded. It was concluded from that study that this metabolite accounts for an insignificant fraction of the administered dose (167). Whether these researchers actually isolated N-acetylphenelzine still needs to be confirmed.

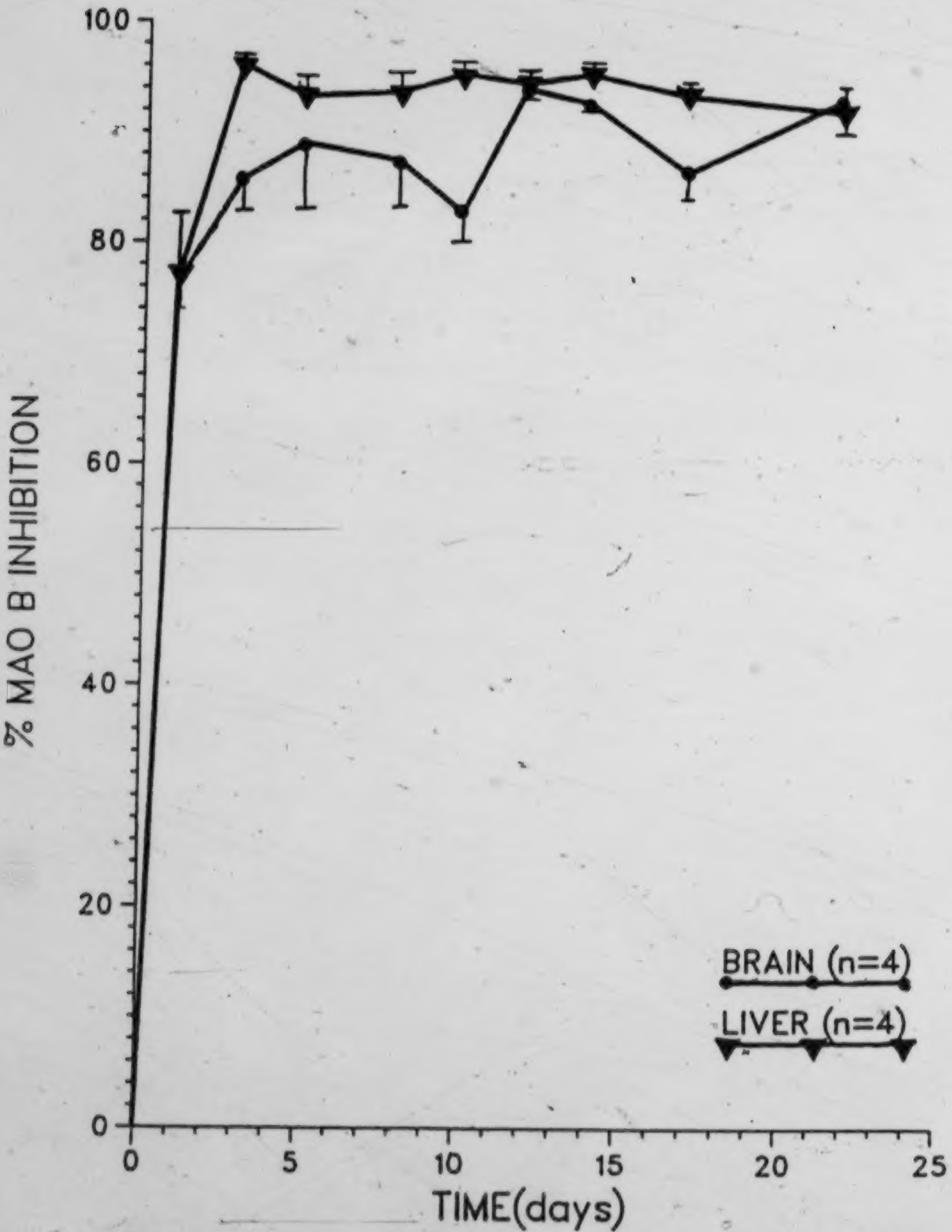
Another PLZ metabolite of current interest to researchers is PEA. Dyck et al. (98) demonstrated that PEA, an endogenous CNS trace amine formed by the decarboxylation of phenylalanine, could also be derived from PLZ. This was confirmed by administering d_4-PLZ (50 mg/kg) and isolating the corresponding d_4-PEA that was formed. The conversion was found to proceed quickly; d_4-PEA was observed in the CNS 20 min after the administration of PLZ. Since PEA is rapidly converted to PAA by the enzyme MAO B (346), its normal level within the CNS is very low (1 ng/g of tissue) (316). However, following the administration the non-selective MAO inhibitor tranylcypromine or the MAO B selective inhibitor deprenyl the brain level of PEA increases rapidly and significantly (322). In contrast, when PLZ, another MAO B inhibitor, was administered to rats, no immediate increase in the brain PEA level was observed. PLZ must be given daily to rats, for 8 consecutive days (16), before increased PEA levels are observed. Continued daily administration of PLZ beyond this 8 day period leads to a further increase in the PEA concentration within the CNS. This delay in the formation of PEA following the administration of a therapeutic dose of PLZ could account for the 2-3 week delay observed before the antidepressant effects of PLZ are observed. PEA is known to have amphetamine-like stimulant properties (330). It has been postulated that increases in PEA may be required for MAOI therapy to be effective (330). Research was undertaken to investigate this unexpected delay in the increase in brain PEA levels following chronic PLZ administration.

3.1.4.1 Effect of Phenelzine on MAO B Activity

Metabolism of PEA by MAO B is the predominant pathway of PEA elimination *in vivo*. An increase in the level of PEA within the CNS is observed only when this enzyme is significantly inhibited. It has been suggested that MAO must be at least 80% inhibited before an increase in the level of the "trace amine" is observed (315). The delayed increase in PEA levels, by the chronic administration of PLZ, might be explained by a corresponding delay in the inhibition of MAO B. Since PEA readily crosses the blood-brain barrier it was necessary to measure the activity of MAO B within the CNS tissue and in peripheral tissues. Several peripheral tissues (for example, blood platelets, liver and lung) possess high MAO B activity (316). Chronic dosing with PLZ may be necessary to significantly inhibit the activity of MAO B in these peripheral tissues. MAO B activity was measured, in both liver and brain tissue, 6 h after a dose of PLZ sulfate (15 mg/kg, free base). The liver was included in the study since this organ contains high levels of this enzyme (317). Following a single dose of PLZ, the activity of MAO B was rapidly inhibited by 76.0 ± 5.0% of control values in both tissues. After administration of PLZ once daily for 3 days, the level of MAO B inhibition increased further to 96.0 ± 1.0% and 85.0 ± 3.0% in liver and brain tissue, respectively. No further increase in MAO B inhibition was observed in either tissue with continued daily administration of PLZ (Fig. 35). In addition to MAO B, the activity of MAO A was also measured in both tissues following a single dose of PLZ. Although MAO A isozyme is not involved in the metabolism of PEA, it is likely to be involved in the metabolism of PLZ itself (98). Since this enzyme could influence the rate PLZ is eliminated from the body the activity of this enzyme was determined following a dose of PLZ. This isozyme was effectively inhibited by 90.0 ± 3.0% in both tissues.

The decrease in MAO B activity did not correlate with the observed delay in the increase of PEA, following PLZ treatment. The levels of PEA within the CNS did not increase until 6 days after maximal MAO B inhibition was attained. The inhibition of MAO B in the liver following PLZ treatment was equal to or greater than the activity of this enzyme in the brain. Since PEA readily crosses the blood brain barrier, it was thought that the PEA formed in the CNS could still be metabolized at a peripheral site, if sufficient peripheral MAO B activity was retained after a dose of PLZ. However, the lack of MAO B activity in the liver suggests that the observed delay in PEA formation following PLZ administration was not due to its metabolism in the periphery. One explanation for the low

Figure 35. Monoamine oxidase B inhibition, in rats, following a single dose of phenelzine or after multiple doses of this drug. The dose of phenelzine administered was 15 mg/kg, calculated as the free base. The procedure used to measure MAO B activity is described in material and methods. (Mean ± S.D.).



CNS level of PEA, even in the presence of significant MAO B inhibition, is that PLZ may also inhibit the formation of PEA from phenylalanine. PLZ administration is known to cause a decrease in the level of pyridoxine (168). It is this cofactor which is required by the enzyme (aromatic amino acid decarboxylase) involved in the formation of PEA from phenylalanine (318).

3.1.4.2 Metabolism of 1-^{14}C-Phenelzine

Excretion of Urinary Phenelzine Metabolites

The conversion of PLZ to PEA observed by Dyck et al. (98) was postulated to proceed through an intermediate N-oxidation step (319). The N-oxidized product that is formed, has never been isolated but was suggested to be N'-hydroxyphenelzine which rearranges to produce PEA. Not only has the conversion of PLZ to PEA been documented, but pheniprazine, which is the hydrazine analog of amphetamine is metabolized to amphetamine (103). The conversion of PLZ to PEA could occur in several organs. N-Oxidations have been documented in several tissues (e.g. liver, lung and kidney) (320). However, the site of PEA formation from PLZ may not be important since PEA readily crosses the blood/brain barrier, gaining access to CNS tissue.

In the study conducted by Dyck et al. (98) d_4-PEA was isolated, from CNS tissue, 20 min after a large single dose of d_4-PLZ (100 mg/kg). Since the rapid increase of PEA following a large dose of PLZ was also demonstrated with unlabelled PLZ (17) it is evident that deuterium labelling had little effect on the results observed. Substitution of a hydrogen atom with a deuterium atom α to an amine reduces the oxidation potential of that compound (8).

In the present study the formation of PEA from PLZ after chronic PLZ treatment was investigated using 1-^{14}C-PLZ. The PEA formed could then be easily detected following its isolation on TLC, since it would retain the ^{14}C-label, and the label would not interfere with the oxidation of PEA to PAA by MAO B. In addition, the use of a radiolabelled tracer (1-^{14}C-PLZ) would permit other metabolites of PLZ to be detected.

Following the administration of 1-^{14}C-PLZ to male Sprague-Dawley rats, 59.2 ± 7.4% of the radioactivity was detected in the urine collected over a 24 h period (Table 5). Neutral and basic compounds were isolated from urine samples by solvent extraction after adjusting the pH to 9.0. PEA and PLZ were separated from other basic materials on TLC plates after being converted to N-ethylchloroformate derivatives. The unextracted fraction (acidic and conjugated compounds) accounted for 90% of the total urinary radioactivity. The neutral fraction represented an additional 1% of the urinary radioactivity, while PEA and PLZ, isolated from the basic fraction represented only 0.01 and 0.05% of the administered dose, respectively. No other basic metabolites were identified. When the neutral fraction was separated on silica gel TLC plates, a significant quantity of the radioactivity present in this fraction was lost. This loss was attributed to the presence of volatile neutral metabolites, including EBZ.

Clineshmidt and Horita (95) have demonstrated that PLZ is converted *in vivo* to PAA. Following the administration of radiolabelled PLZ to rats, it was observed that a significant portion of the urinary radioactivity (70% of the dose) was present as PAA in the acid extractable fraction. The acid extractable fraction in their study corresponds to the unextracted fraction of the present study.

In the present study, changes in the pattern of PLZ metabolism were observed when PLZ was administered daily for 15 days. The total radioactivity detected in 24 h urine was significantly reduced (41% of the dose). This reduction coincided with a 2-fold increase in the urinary excretion of neutral metabolites and a 4-fold increase in the quantity of PEA and PLZ eliminated in urine (Table 5). After chronic PLZ treatment, the unextracted fraction which remained after the isolation of bases and

Table 5. The effects of prior phenelzine sulfate treatment of rats on the urinary excretion of radioactivity during the 24 hours following an injection of 1-^{14}C-phenelzine sulfate. (Mean ± S.D., N=4).

	No Prior Treatment with "cold" phenelzine sulfate	Prior treatment with "cold" phenelzine sulfate
Total Urinary Radioactivity DPM X 10^3	2600 ± 320 (59.2 ± 7.40)[a]	1800 ± 500[c] (41.0 ± 11.2)[a]
Unextracted[b] Fraction DPM X 10^3	2300 ± 210	1600 ± 400[c]
Basic Fraction[b] DPM X 10^3		
Phenelzine	1.1 ± 0.54	4.1 ± 1.3[c]
Phenylethylamine	0.25 ± 0.15	1.2 ± 0.65[c]
Neutral Fraction[b] DPM X 10^3	26.60 ± 5.7	44.4 ± 21.0[c]

a- percentage of dose ± S.D.

b- the efficiency of recovery was not determined

c- differs significantly from data obtained after a single dose, $P<0.05$.

neutrals from the urine, was still believed to contain significant quantities of PAA, even though the activities of MAO A and B were significantly reduced. Clineschmidt and Horita (95) observed a similar decrease in the amount of radioactivity excreted in urine when they administered 1-^{14}C-PLZ to rats pretreated with tranylcypromine or pargyline. A significant quantity of the urinary radioactivity was still associated with PAA, isolated by TLC. Since MAO is significantly inhibited after both chronic PLZ treatment or a single dose of tranylcypromine, the metabolism of PLZ to PAA may also be mediated by another enzyme, possibly diamine oxidase. It has been previously shown that tranylcypromine and pargyline are weak inhibitors of this enzyme, while PLZ competitively inhibits diamine oxidase (321).

The increased urinary excretion of PLZ observed after chronic PLZ administration to rats was also observed when PLZ was given chronically to human volunteers (162). The results obtained in that study could be questioned since the analytical procedure used could not differentiate between PLZ and those metabolites (N-hydroxyphenelzine, N-acetylphenelzine) which retained the hydrazine moiety.

Only 0.01% of the PLZ administered as a single dose was recovered in 24 h urine as PEA. The amount of PEA recovered in 24 h urine increased 4-fold when 1-^{14}C-PLZ was administered to rats pretreated with cold PLZ for 14 days. The conversion of PLZ to PEA, even after chronic PLZ treatment, appears to be a minor route of PLZ metabolism. Following the administration of other MAOIs (e.g. tranylcypromine) significant increases in both urinary and CNS PEA levels have been observed (322,323). This occurs since PEA, which is normally eliminated by metabolism to PAA, can also be eliminated by urinary excretion when the metabolic pathway to PAA is impaired. The 4-fold increase in radiolabelled PEA observed in urine following chronic PLZ administration does not correlate with the 150-fold increase in the level of PEA observed in the CNS following a similar PLZ dosing schedule. In a previous study (98) when brain levels of PEA rose to 250 ng/g, in response to a large dose of PLZ (50 mg/kg), a significant quantity of PEA (100 ug) was found in urine, collected for 24 h after drug administration. In this current study, however, only small amounts of PEA (1.5 ug) could be isolated from 24 h urine, after chronic PLZ administration. Following chronic PLZ administration to rats, the brain level of PEA are known to increase significantly (greater than 150 ng/g). The quantity of PEA isolated in the 24 h urine did not correlate with the increase in brain PEA levels observed following chronic PLZ administration.

Expiration of Radioactivity

Following the administration of 1-^{14}C-PLZ to rats, radioactivity was detected in the expired air of these animals. The expired volatile metabolite(s) was (were) isolated by drawing the expired air through a solution of 2-methoxyflurane/ethanolamine (3:1) for a 24 h period. The level of radioactivity in the trapping solution was determined at regular intervals by liquid scintillation counting. Rats which were pretreated with cold PLZ prior to the administration of the radiolabelled PLZ had significantly greater (4-fold increase) expiration of radioactivity than did rats with no prior PLZ treatment. The quantity of trapped radioactivity did not reach a plateau as one would expect in an accumulation study, but rather rapidly declined 4 h after collection of the expired radioactivity was initiated (Fig. 36). This suggests that a volatile metabolite, other than carbon dioxide, which was trapped by the methoxyflurane/ethanolamine solution was lost during the prolonged collection period. Since the carbon dioxide is chemically trapped in this solution little would be lost (324). Very little radiolabelled carbon dioxide was expired by PLZ treated rats.

Although only 2% of the administered 1-^{14}C-PLZ dose was isolated in the expired air of rats pretreated with cold PLZ, it is evident that a greater quantity than 2% of volatile metabolite(s) was actually excreted. Peak expiration of radioactivity was observed 4 h after the administration of 1-^{14}C-PLZ. Attempts to trap this metabolite

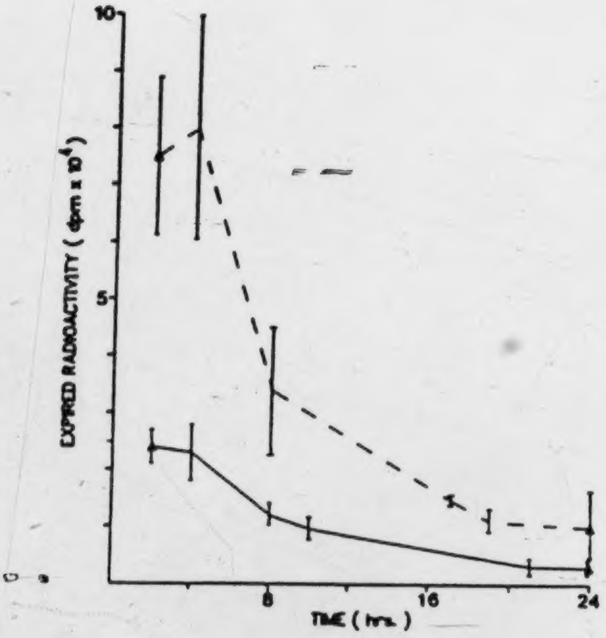


Figure 36. The effects of chronic phenelzine sulfate treatment on the expired radioactivity after a dose of 1-^{14}C-phenelzine in the rat. (Mean ± S.D.). All values obtained from animals pretreated with phenelzine sulfate differed from those animals which had no prior drug treatment ($P<0.05$).

No prior drug treatment ——

Prior drug treatment. ----

quantitatively are discussed in section 3.

Mass spectrometry studies revealed that the trapped volatile product excreted in the expired air of rats was EBZ (Fig 37). This metabolite, previously shown to be an *in vitro* metabolite of PLZ, is believed to be formed by either a free radical process (13) or via a metabolic N-oxidation (325). The conversion of PLZ to the N-oxidized product is believed to be mediated by the enzyme alkylhydrazine oxidase. Both free radicals and N-oxidized compounds are toxic, *in vivo* (326). PLZ administration is known to induce tumors in mice (327). The tumors observed may be a result of the formation of EBZ by either metabolic route.

Elimination of volatile metabolites in expired air is relatively rare except for some gases (e.g. carbon dioxide or inhalation anesthetics) which are eliminated by this route (328). It has also been shown that volatile liquids could also be excreted in expired air. The presence of toluene in the expired air of people exposed to this solvent has been demonstrated (198). However, only a few studies have demonstrated the expiration of a volatile metabolite, other than carbon dioxide, of drugs or xenobiotics. Besides the excretion of EBZ, demonstrated in this study, propane has been found to be a volatile metabolite of iproniazid, and it is excreted in expired air (107). Isolation of volatile metabolites in expired air provides evidence that the lung can participate in the excretion of xenobiotics. The role the lung plays in drug elimination requires further investigation to fully evaluate its potential as an excretory organ.

Residual Tissue Radioactivity

Since only 63% of a dose of PLZ could be recovered from rat urine and expired air in a 24 h period, the level of radioactivity which remained in the tissues was determined. It is possible that a significant quantity of the administered PLZ dose remains covalently bound to tissue constituents. If true this could result in severe toxic side effects. Several organs (liver, lung, kidney, spleen, heart and brain) were removed, digested in Protosol® and their remaining radioactivity determined by liquid

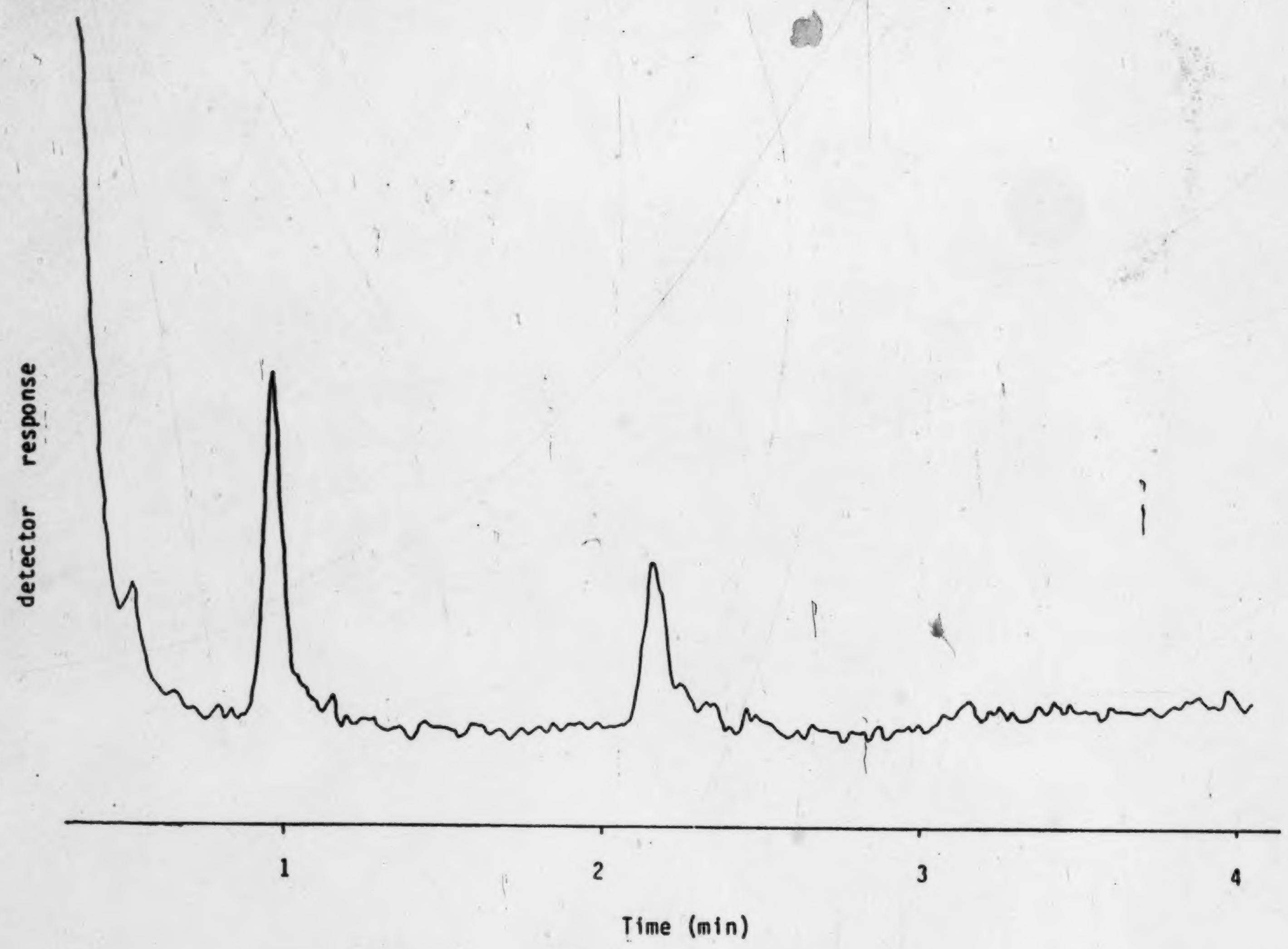


Figure 37. The total ion trace of the volatiles isolated from the expired air of rats treated with phenelzine sulfate (15 mg/kg, free base). The retention time of ethylbenzene is 1.0 min. The other GC peak was a endogenous compound.

scintillation counting (Table 6). All the samples counted were corrected for quenching using the sample channel ratio method.

The radioactivity present in these tissues, 24 h after a dose of 1-^{14}C-PLZ, accounted for only 0.7% of the administered dose. It is evident that the 37% of the dose unaccounted for was not retained in the tissues examined.

3.1.4.3 Rat Liver Perfusion Studies

It was evident from the (*in vivo*) radiotracer studies just described that the quantity of a PLZ dose converted to PEA was very low, even when PLZ was administered chronically for 15 days. Dyck et al. (98), however, found that in rats a significant quantities of PEA could be formed from PLZ when this drug was administered at high levels (50 mg/kg). These different observations were worthy of further investigation to determine if the formation of PEA from PLZ was dose-dependent. PEA may not be formed to any great extent unless other pathways of PLZ metabolism are saturated. The isolated rat liver perfusion technique was selected since higher concentrations of PLZ than those given in an *in vivo* experiment can be administered (251). The presence of toxic side effects with PLZ limits the quantity of drug which can be administered *in vivo*. Any PEA detected would be derived only from PLZ since the formation of PEA from phenylalanine would be unlikely to occur with this preparation. In addition, the preparation could be used to simulate the liver metabolism of PLZ after chronic dosing simply by repeating the perfusion experiment using livers isolated from rats pretreated with PLZ. The liver was an appropriate organ to investigate PLZ conversion to PEA since this organ does possess the enzymes which mediate N-oxidation, the metabolic pathway postulated from microbial studies to be involved in this conversion (319).

PLZ (3.14 or 16.2 ug/ml) dissolved in Kreb's bicarbonate buffer was infused into the isolated liver preparation, for an 80 min period. Prior to infusing the drug, the liver was perfused with Kreb's bicarbonate buffer alone, for a 30 min period. Preliminary studies with this preparation demonstrated that little PEA was formed even when PLZ was

Table 6. Radioactivity present in selected tissue 24 h after an acute dose of 1-^{14}C-phenelzine (Mean ± S.D., N=4).

Tissue	DPM
Liver	26314 ± 2286
Kidney	5587 ± 762
Brain	552 ± 194
Heart	102 ± 45
Spleen	138 ± 66
Lung	501 ± 141
Total*	33194

* 0.7% of the dose.

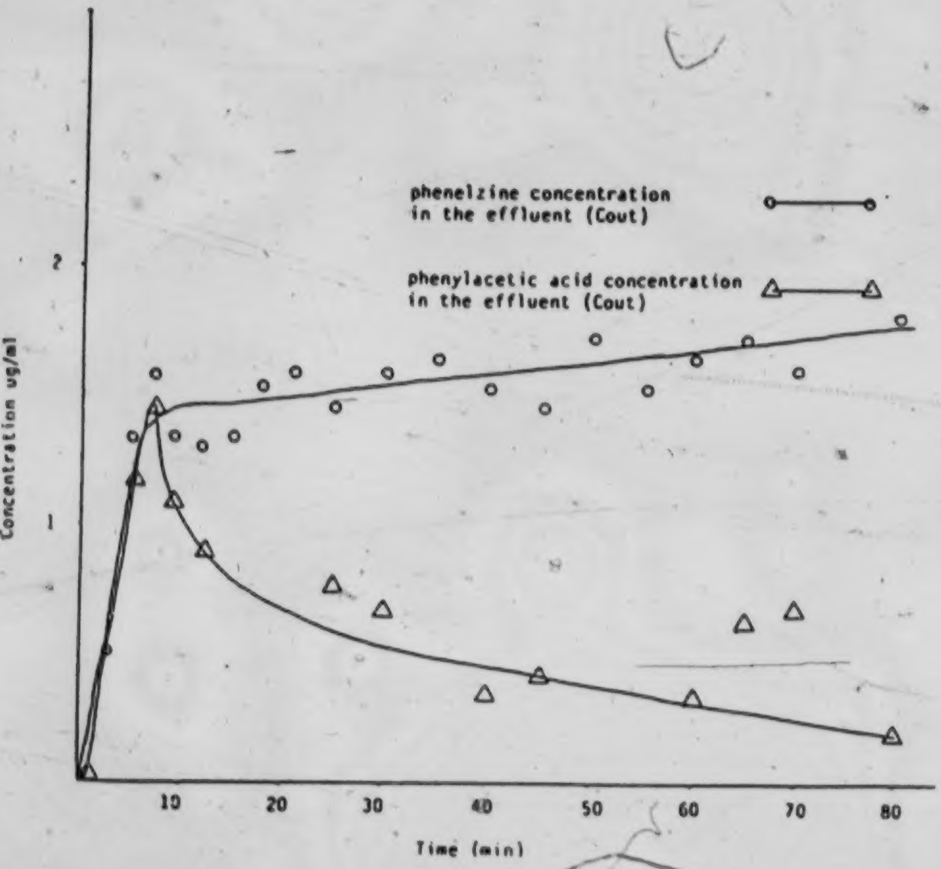
infused at high concentrations (16.2 ug/ml). The liver was metabolically active since PLZ could be converted to PAA in this preparation during the first 10 min of infusion (Fig. 38). The decrease in the concentration of PAA in the effluent after this time period was attributed to the inhibition of the liver MAO enzyme, since oxygen consumption and the perfusion flow rate remained constant throughout the experiment. Both of these parameters were used to monitor liver viability. Changes in the livers vascular resistance would be reflected by an increase in the perfusate reservoir.

The concentration of PLZ in the effluent never reached steady state (Fig. 39). This inability to attain a steady state concentration in the effluent using the "once through" perfusion technique indicates that during PLZ infusion, a reduction occurred in the metabolic elimination of this drug which could be attributed to the inhibition of enzymes responsible for the elimination of PLZ (e.g. MAO). This inability to reach steady state was even more pronounced when PLZ was infused at a higher concentration. At this higher infusion concentration, there was an obvious reduction in the elimination of PLZ, yet the quantity of PEA formed was still below the detectable limit of the assay (20 ng/ml). This is surprising since, in rats, significant quantities of PEA were formed when a large dose of PLZ was administered. It is also possible that the inability to detect PEA when PLZ was infused at this high concentration is due to a PLZ-induced hepatotoxicity. PLZ is known to be hepatotoxic *in vivo* (110). However, again both oxygen consumption and perfusion flow rates (20-25 ml/min) were constant throughout the experiment, indicating no obvious liver damage.

N'-Acetylphenelzine, a proposed metabolite of PLZ, was not observed in the effluent obtained from livers perfused with PLZ. This compound if present at a cocentration above 50 ng/ml of effluent, would have been detected by the analytical procedure used in this study.

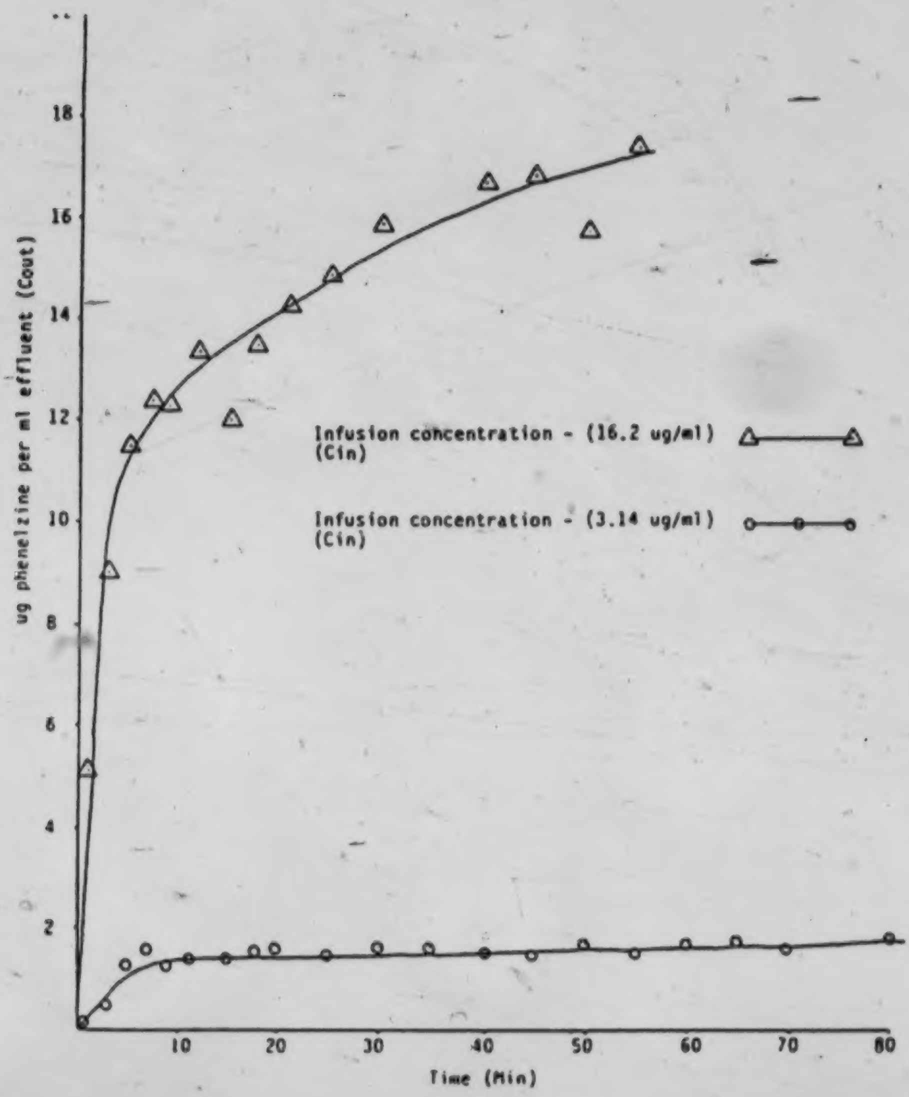
The hypothesis that the formation of PEA, after a single dose of PLZ, was only observed when this drug was given at a high concentration may not be true. The theory

Figure 38. The concentration of phenelzine and phenylacetic acid in the effluent obtained by perfusing an isolated rat liver with phenelzine dissolved in Kreb's bicarbonate buffer. The drug was infused using the "once through" perfusion method.



-perfusion flow rate 25 ml/min
-oxygen consumption 80%
-N=1

Figure 39. Concentration of phenelzine in the effluent obtained when isolated rat livers were infused with this drug. The samples were withdrawn at predetermined time intervals. The drug was infused by the "once through" perfusion method.



N=2

implies that following saturation of other routes of PLZ elimination, PLZ would then be converted to PEA. However, this was not observed in the isolated liver perfusion study, yet it was evident, particularly at the higher infusion concentration, that a reduction in the elimination of PLZ had occurred and presumably saturation of normal metabolic pathways had resulted. Whether the dose dependant formation of PEA, from PLZ, occurs *in vivo* must be further investigated.

3.1.4.4 Pharmacokinetics of Phenelzine

To help explain the change in the metabolism of PLZ observed with chronic dosing, blood concentration-time curves were generated following a single dose or after multiple daily doses of PLZ. In addition, the blood level of PEA was also measured at several times following the administration of PLZ to rats which were either drug naive or pretreated for several days with PLZ. The incentive to conduct this research was due predominantly to the different observations made in the studies conducted by Baker et al. (16) and Dyck et al. (98) on the formation of PEA following a dose of PLZ. In the first study no increase in PEA was observed unless PLZ was administered chronically for 8 days (once a day). The dose used in that particular study was 15 mg/kg, and the CNS level of PEA was not measured until 6 h after the dose. Dyck et al. (98) and Philips et al. (17) were able to detect PEA, in the brain, 20 and 30 min, respectively, after an acute dose of PLZ. In these two studies a significantly larger dose of PLZ was administered (50-100 mg/kg). In light of these observations it was important to follow the time course of PEA in the body, not only after a single dose of PLZ, but also after chronic PLZ administration. In this study no attempt was made to differentiate the source of PEA (i.e. PLZ or phenylalanine). Rather the study was designed to obtain an overall picture of PEA formation from PLZ or phenylalanine after chronic PLZ dosing. The level of PLZ or PEA was measured in blood since the time course of a compound in the body can be easily demonstrated in this fluid (329). The blood was used rather than plasma so that the total blood concentration could be determined (both protein bound and unbound). Since PEA readily crosses the blood-brain barrier, increases in the level of PEA in the brain should be associated with an increase in the level of PEA in the blood (316).

Blood concentration-time curves were generated by measuring the PLZ concentration in blood removed through a jugular vein cannula at several predetermined times following the i.v. administration of PLZ (15 mg/kg, free base) to rats. These curves were generated after the 1st, 7th and 9th dose of PLZ, administered once daily (Fig. 40). Prior to administration of the drug, a blank blood sample was withdrawn. PLZ was administered i.v. to permit calculation of ClTB and T1/2 (Appendix A and B, respectively). The experiments could only be conducted over a 9 day period. After this time, clotting occurred. The exposed cannula was filled with a sodium citrate block after blood sampling. Heparin might have been a better anticoagulant but its use was avoided since this compound can influence the kinetics of basic drugs, by altering their serum protein binding (331). With chronic administration of PLZ, the half-life of this drug increased from 72 to 187 min by the 7th dose. No further increase in T1/2 was observed with continued daily administration of PLZ (Table 7). This change in T1/2 was associated with a decrease in the ClTB. Between the 7th and 9th dose, the ClTB was even further decreased. With prolonged PLZ administration, the conversion of PLZ to EBZ, other neutral metabolites and PEA increased in importance.

The changes in the elimination of PLZ were not solely time-dependent. The clearance of PLZ could also be reduced by administering a large single dose of this drug (Fig 41). The T1/2 of PLZ increased from 70.4 to 103.2 min and the ClTB decreased from 1.3 to 0.38 l/h when the dose of PLZ was increased from 15 to 30 mg/kg, both calculated as the free base (Table 8). No further change in T1/2 or ClTB was observed when the dose was increased from 30 to 45 mg/kg. The dose of PLZ administered to rats by Dyck et al. (98) in their study was even larger than the dose of PLZ used in this study. This suggests that the formation of PEA they observed occurred when other pathways of PLZ metabolism were either saturated or inhibited.

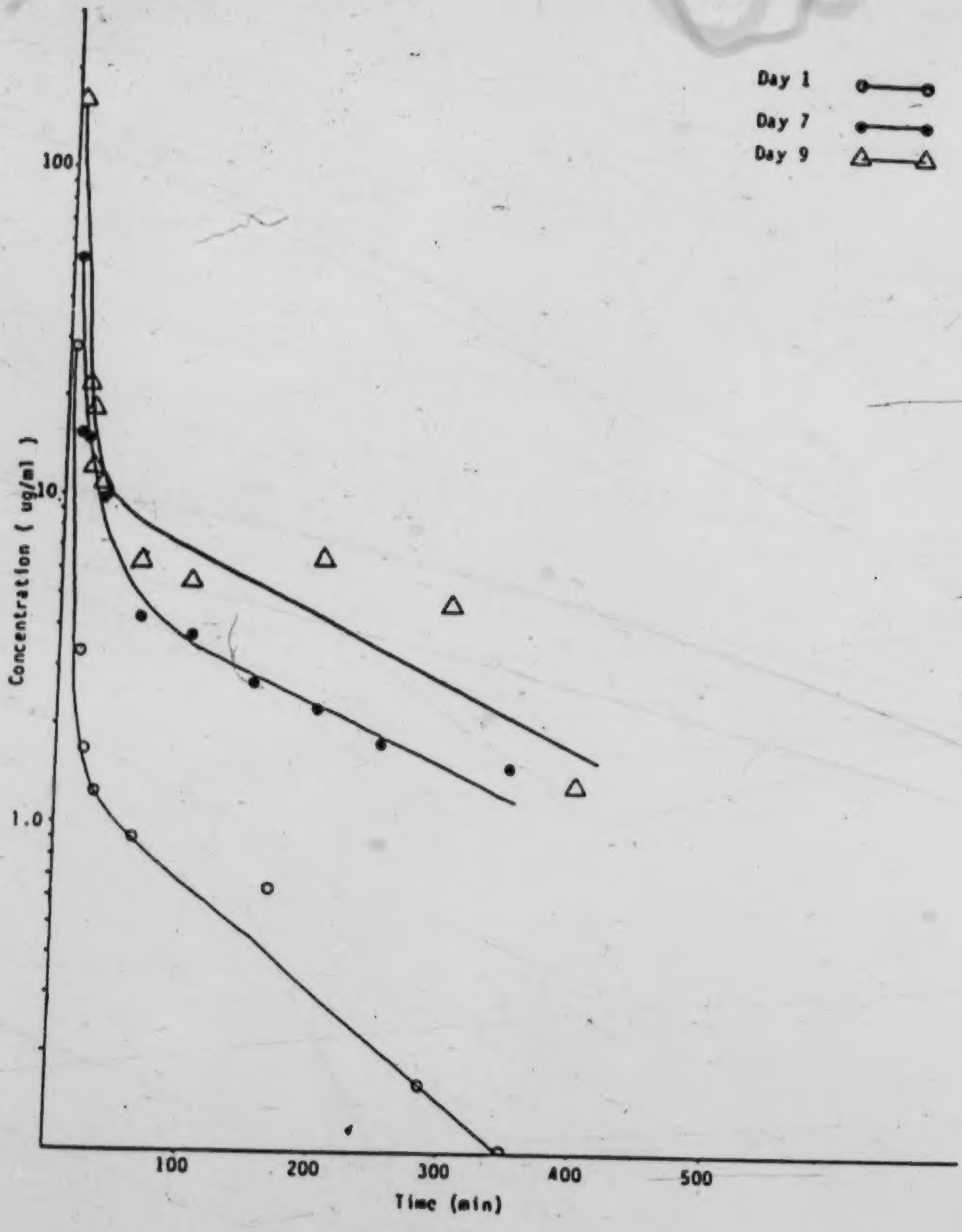


Figure 40. The blood concentration-time curve of phenelzine following single or multiple doses (once a day) of this drug. The dose administered was 15 mg/kg. calculated as the free base. The blood samples were withdrawn after the last dose was administered. (Mean, N=3).

Table 7. Various pharmacokinetic parameters (Mean ± S.D., N=6) calculated from the blood concentration-time curves of phenelzine, obtained when the blood level of phenelzine was measured at several time intervals following administration of this drug to rats (15 mg/kg, Free base).

	α^1	β^1	$AUC_{5-\infty}^2$	Cl_{TB}^3	$T1/2^4$	Vd^5
Day 1	0.052±0.04	0.011±0.004	0.29±0.05	1.3±0.25	70.4±28.6	2.2
Day 7	0.20±0.06	0.0039±0.0009	1.73±0.11	0.22±0.02	182.0±41.0	0.96
Day 9	0.52±0.16	0.0041±0.0006	3.20±1.34	0.13±0.04	171.0±23.5	0.53

1. min^{-1}
2. mg/ml/min
3. L/h
4. min
5. L

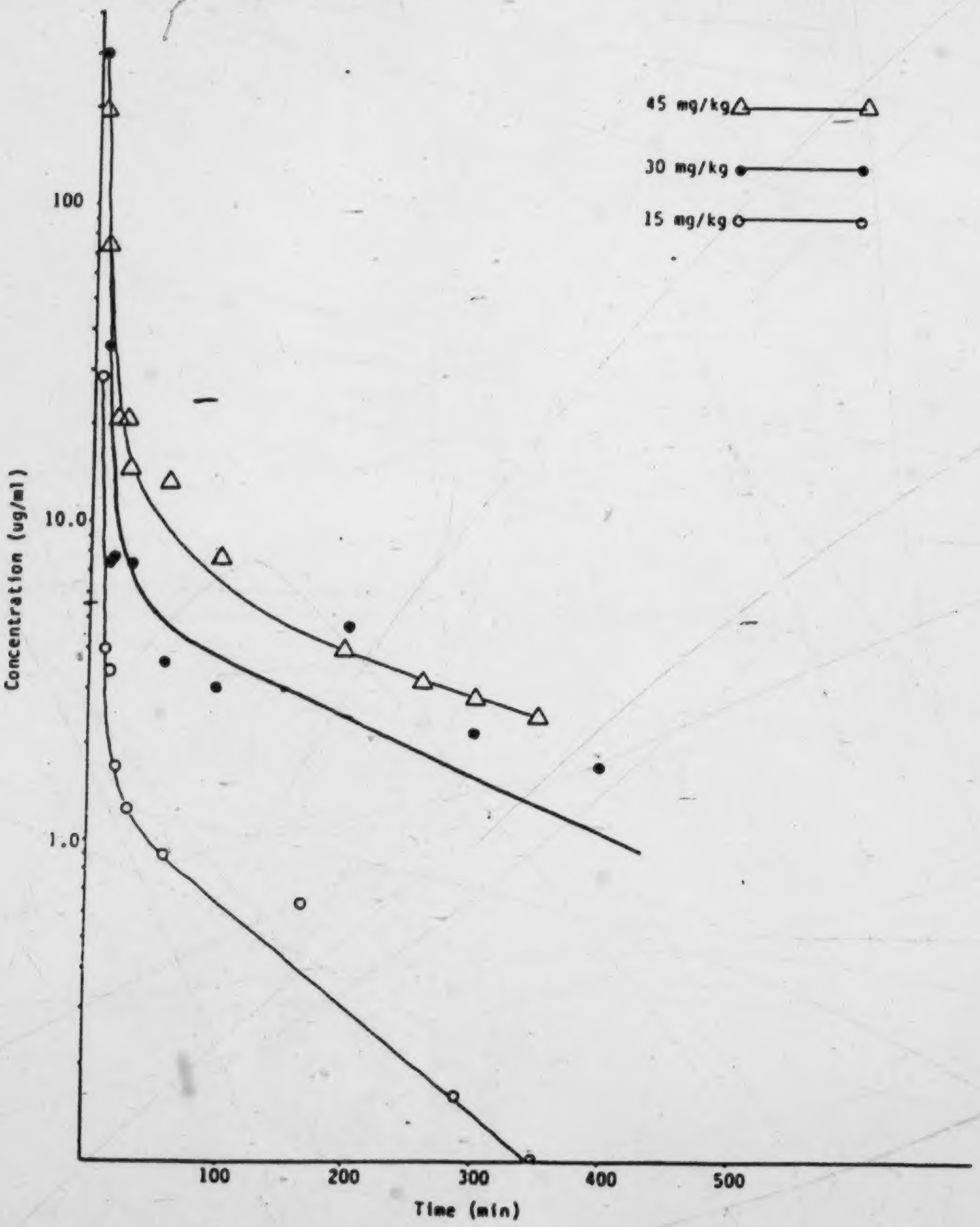


Figure 41. The blood concentration-time curves of phenelzine obtained when rats were administered single doses of this drug (15, 30 or 45 mg/kg, free base). (Mean, N=6)

Table 8. Various pharmacokinetic parameters (Mean±S.D., N=6) calculated from the blood concentration-time curves obtained when the blood level of phenelzine was measured, in rats, at several time intervals following the administration of this drug, intravenously. The dose of phenelzine administered was either 15, 30 or 45 mg/kg, calculated as the free base.

	α^1	β^1	$AUC^2_{5-\infty}$	Cl^3_{TB}	$T1/2^4$	Vd^5
15 mg/kg	0.52±0.04	0.011±0.004	0.29±0.05	1.3±0.25	70.4±28.6	2.2
30 mg/kg	0.55±0.12	0.0069±0.002	2.61±1.62	0.38±0.22	103.2±32.4	0.94
45 mg/kg	0.47±0.23	0.0077±0.0028	4.20±2.1	0.37±0.24	101.0±28.7	0.89

1. min^{-1}
2. mg/ml/min
3. L/h
4. min
5. L

3.1.4.5 The Effect of Phenelzine Administration on Phenylethylamine

The influence of chronic PLZ administration on the formation of PEA was determined by measuring the blood level of PEA at regular intervals over a 7 h period following the dose of PLZ. The blood level of PEA rapidly increased to 70 ng/ml of blood during the first 10 min following the administration of PLZ (15 mg/kg) to drug naive rats. The PEA formed, however, was quickly eliminated from the blood after this time period, probably by renal excretion, since MAO B is rapidly inhibited by PLZ administration. When PLZ was administered daily over a 9 day period there was an increase in the quantity of PEA detected in blood (Fig 42). This increase could be due either to an increase in its formation or to a reduction in the elimination of PEA. If the latter situation is true the impairment of PEA elimination is bleived to result from a reduction in its renal excretion, and not to a decrease in its metabolism. MAO B, the enzyme responsible for the metabolism of PEA, is rapidly inhibited following PLZ administration and other major pathways of PEA metabolism have not been observed.

3.1.4.6 Kidney Damage Induced by Phenelzine

Preliminary investigations of rats chronically dosed (i.v.) with PLZ have indicated that in some animals this drug induced kidney damage. This was demonstrated by the presence of casts and red cells in the urine of these animals. The effect of the route of administration on the kidney damage was not investigated. However, even chronic PLZ administration by the i.p. route appears to have resulted in kidney damage. Kidneys obtained from rats dosed with PLZ for 15 days weighed significantly less than kidneys isolated from control animals dosed with saline for the same period. In addition the lack of PEA in the urine of rats chronically treated with PLZ (for 15 days) where the CNS levels of PEA are known to be high, supports the theory that the renal excretion of PEA is impaired. To determine the site of renal damage would require histological examination of the damaged kidneys. This is the obvious next step in the study of PEA formation following chronic PLZ administration. It should be noted that decreased elimination of

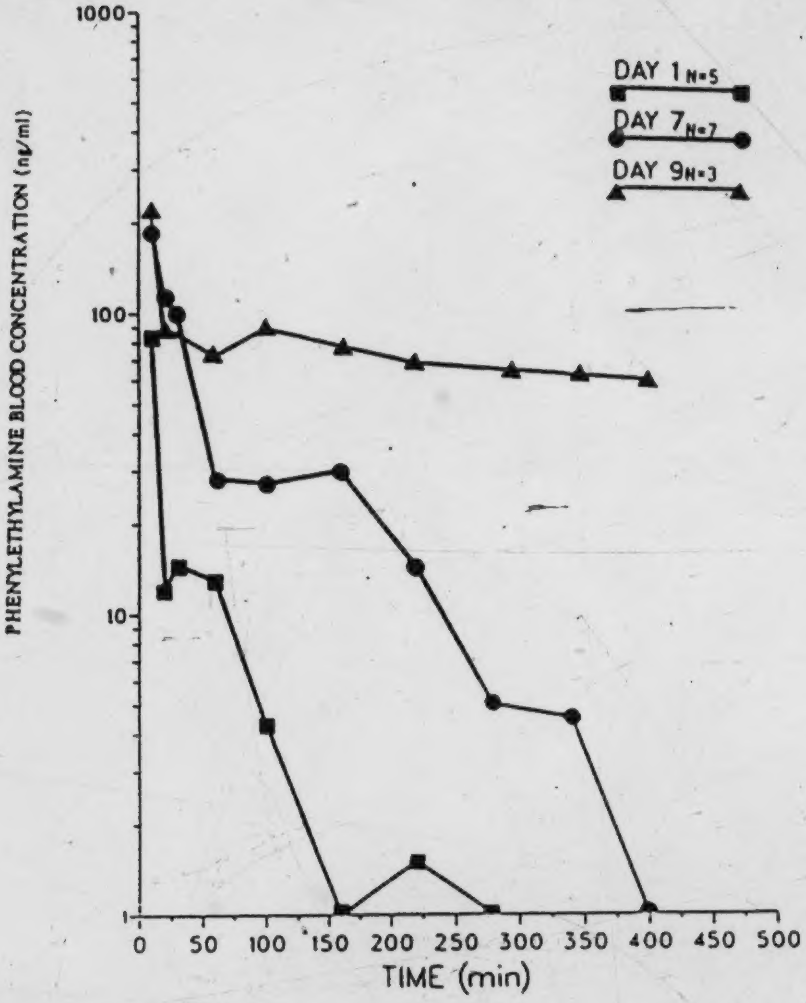


Figure 42. The blood concentration of PEA in rats following the administration of phenelzine sulfate (15 mg/kg, calculated as the free base). The animals were either drug naive or pretreated with phenelzine for 7 or 9 days. (Mean). The AUC for the day 9 experiment was significantly different from the AUC calculated for the day 7 experiment ($P < 0.05$).

PEA does not necessarily indicate significant renal damage. Rather reduced PEA elimination could be associated with damage of amine transport mechanisms which are known to exist in the kidneys (295). A toxic metabolite of PLZ, hydrazine, is known to be eliminated, in part, by renal excretion (332).

3.1.4.7 Biliary Excretion of Phenelzine

The biliary excretion of PLZ was investigated since almost 37% of a single dose of PLZ could not be accounted for by urinary excretion or excretion into expired air. This increased to almost 60% of the dose unaccounted for when rats were pretreated with PLZ for a 15 day period. The quantity of radioactivity present in feces was not measured since this required sample oxidation, a technique unavailable for this study. Evidence from blood concentration-time curves of some animals dosed with PLZ suggested that PLZ could undergo some enterohepatic recirculation. An increase in the blood concentration of PLZ was observed during the elimination phase. Two drugs related in structure to PLZ, pivhydrazine and mebanazine, were both found to be excreted into bile of rats (89). It was suggested that these compounds were conjugated to glucuronic acid forming N-glucuronides, prior to their elimination into bile. This conclusion was based on the observation that the conjugates could be easily hydrolyzed with either β-glucuronidase or in acetate buffer (pH 5.5). It is conceivable that PLZ could also be eliminated in bile.

In the present study the biliary excretion of PLZ was investigated using bile duct-cannulated rats. The concentration of PLZ was measured in bile rather than feces. Since the microflora present in the gastrointestinal tract can metabolize drugs, the presence of xenobiotics in the feces does not signify they were present in the body. For this reason the excretion of PLZ into bile and not feces was investigated. In addition the study of bile would also permit experiments to be designed to investigate the enterohepatic recirculation of this drug. This can be achieved by comparing the AUC for bile duct-cannulated rats administered PLZ to the AUC determined for sham-operated animals. In addition, the sham-operated animals were used to ensure that the surgical procedure did not influence the kinetics of PLZ.

To investigate the biliary excretion of PLZ in rats a new bile duct-cannulation procedure was developed. Previously reported methods have utilized anesthetized animals immediately following surgery (284) or after regaining consciousness (272). In the latter procedure, the animals, once they had recovered, were maintained in a restraining device throughout the duration of the experiment. Anesthetic agents, decreases in blood pressure and hypothermia can all reduce bile flow and therefore the biliary excretion of a drug (274,276). Animals allowed to recover from anesthesia, but maintained in a restraining device, suffer from hypothermia. In these experiments the body temperatures of the rats had to be maintained using an incandescent lamp (272,283,287). In addition, anesthetics including pentobarbital, reduced both glucuronide and sulfate conjugation, the forms in which most xenobiotics are eliminated in bile (288).

Two methods have been developed which allow collection of bile from freely moving animals (278,293). In both procedures bile was continuously collected once the bile duct had been cannulated. A means of recycling bile was not permitted by these techniques. Besides the obvious dehydration which can result from continuously collecting bile, loss of bile salts will cause a reduction in bile flow (277). Even replacement of the fluid lost during bile collection with a saline solution (0.9%) will not re-establish normal bile flow. Infusion of sodium taurocholate has been used to stimulate bile salt production and thereby increase bile flow. However, if this method is used, the rate at which sodium taurocholate is infused must be strictly controlled since choleresis (overstimulation of bile flow) can occur, if this compound was infused too rapidly (277).

The procedure developed here for the study of biliary excretion offers several advantages over current techniques:

1. Animals are allowed to recover for 1 day from surgical and anesthetic procedures, prior to the experiment.
2. During recovery bile is allowed to recycle normally so that bile salts are conserved. This

ensures that normal bile flow is attained at the start of the biliary excretion study.

3. Following the cannulation procedure, animals are housed in conventional plexi-glass cages. During the experiments the animals can be maintained in these conventional cages or easily transferred to metabolic cages for the collection of urine, feces and expired air, in addition to bile.
4. Exteriorization of the bile duct cannula is greatly simplified and no longer requires sophisticated apparatus (e.g. swivel joints) to maintain animals for long periods of time.
5. Following the experiment, normal recycling of bile can be easily re-established. This allows rats to be reused for biliary excretion studies (e.g. chronic studies).

Bile duct-cannulated rats were used in two separate experiments over a 7 day period. Bile flow was similar in both experiments. During the recovery period, bile was allowed to recycle by virtue of a re-entrant cannula. One day following surgery, the cannula was disconnected and bile collected at predetermined time intervals over a 7 h period. The bile flow rate was measured from these samples. Initially bile flow rate was 27.0 ± 8.0 ul/min. After 2 h the bile flow rate began to decrease and reached 14.0 ± 5.0 ul/min after 6 h (Table 9). This decrease in bile flow is believed to result from the loss of bile salts which occurs during the collection period (283). The initial bile flow rate obtained in this study was similar to, if not better than, previously reported values (277,294). Methods previously used to measure bile flow utilized animals with an already depleted store of bile salts or still suffering from the effects of surgery. The decrease in bile flow observed during the prolonged collection period did not influence the elimination rate of PLZ (either free or as a labile conjugate) into bile.

The kinetics of PLZ were not affected by the surgical procedure. The T1/2 and ClTB calculated for bile duct-cannulated and sham-operated animals which received a single dose of PLZ (30 mg/kg) were identical to the values obtained for control animals (these animals only had a jugular vein cannula) receiving the same dose of PLZ (Table 10). The blood concentration-time curves generated for each group of animals (bile duct-cannulated, sham-operated and control animals) were almost identical (Fig 43). The values of A, B, α and

Table 9. Rat bile flow rates determined when bile was collected continuously for a 7 hour period. The experiment was conducted on the day following the bile duct cannulation procedure. The flow rate was measured at several time intervals during this 7 h period. These rats had received a dose of phenelzine (30 mg/kg) prior to bile collection. (Mean±S.D., N=6)

Time (min)	0-30	30-50	50-150	150-210	210-270	270-370	370-430
volume (ul/min)	27±8	27±5	26±5	18±4	16±5	16±8	14±5

β, generated from the blood concentration-time curves obtained when PLZ was administered to these animals, were similar to the values obtained from control animals (Table 10). The overall elimination rate constant (0.005 min^{-1}) calculated from the slope of the curve obtained from the PLZ biliary excretion rate plot (Fig 44) was virtually identical to the value of β calculated from the blood concentration-time curves of both bile duct-cannulated (0.007 min^{-1}), sham-operated (0.006 min^{-1}) and control (0.007 min^{-1}) animals. Changes in the value of K (calculated from the slope of biliary excretion rate plot) would be expected if the surgical or collection procedure influenced the kinetics of PLZ.

The total amount of PLZ recovered in bile, collected for a 7 h period, was 11.2 ± 3.9% of the i.v. administered dose (Fig 45). Maximal biliary excretion was observed immediately following administration of the drug. When biliary excretion rates of PLZ were plotted versus time, a linear relationship was observed except at high PLZ blood concentrations (greater than 10 ug/ml of blood) (Fig 44). It has been demonstrated previously that the biliary excretion of a number of other drugs is a saturable process (333).

It is possible that PLZ was eliminated in bile in an unconjugated form. If it was conjugated, the conjugate would have to be extremely labile. After treatment of bile with 0.1N perchloric acid for only 15 min at room temperature, the PLZ was in free form. Possible candidates for conjugates are N-glucuronides or a hydrazone formed by interaction with an endogenous compound (e.g. α-ketoglutarate, pyruvic acid or pyridoxal). N-Glucuronides and hydrazones are readily hydrolyzed in weakly acidic solutions (89,90).

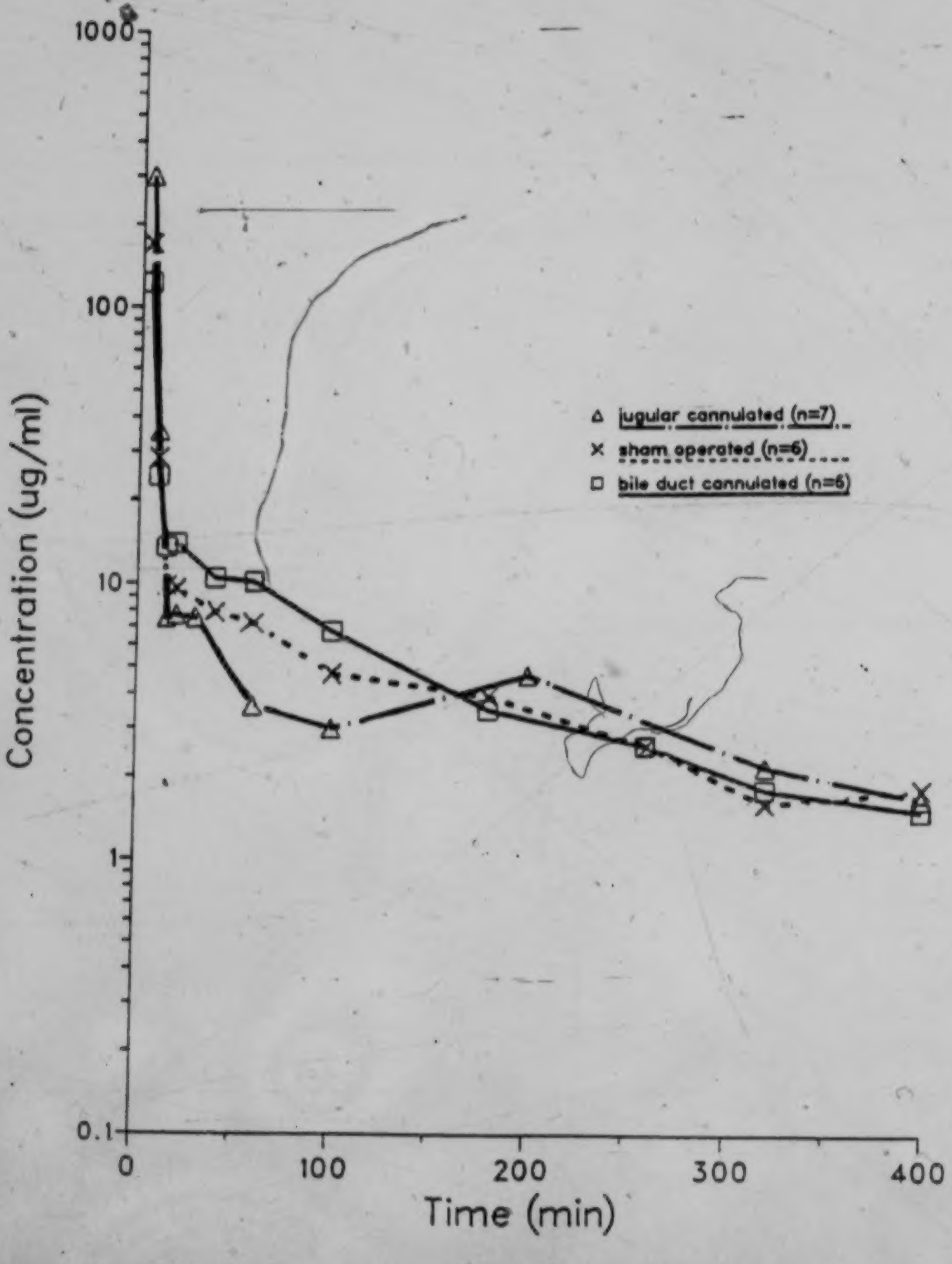
In bile, following hydrolysis (6N HCl, 100°C for 1 h), PLZ was quantitated as its aqueous acetylated/PFPA derivative. To confirm the identity of PLZ, rat bile was treated with the derivatizing reagent PFBC. The pentafluorobenzoylated product isolated by solvent extraction had the same GC retention time as authentic PLZ derivatized with this reagent. N'-acetylphenelzine was not observed in the bile of these animals, following derivatization with PFBC. The observation that N'-acetylphenelzine is not in the bile of rats administered PLZ further substantiates the theory that this compound is not an *in vivo* metabolite of PLZ.

Table 10. Pharmacokinetic parameters (Mean ± S.D., N=6) calculated from the blood concentration-time curves of phenelzine in rats administered this drug intravenously. The drug was given to bile duct-cannulated and sham-operated rats at a dose of 30 mg/kg, calculated as the free base.

	α^1	β^1	$AUC^2_{5-\infty}$	Cl^3_{TB}	$T1/2^4$	Vd^5
Bile duct-cannulated	0.48±0.24	0.007±0.002	2.3±0.71	0.34±0.09	109±33.1	0.89
Sham-operated	0.47±0.10	0.006±0.003	2.6± 1.2	0.36±0.19	151.9±118	1.3

1. min^{-1}
2. mg/ml/min
3. L/h
4. min
5. L

Figure 43. The blood concentration-time curve of phenelzine generated from either control, sham-operated or bile-duct cannulated rats administered this drug (30 mg/kg, free base). The control animals had only a jugular vein cannula. (N=6).



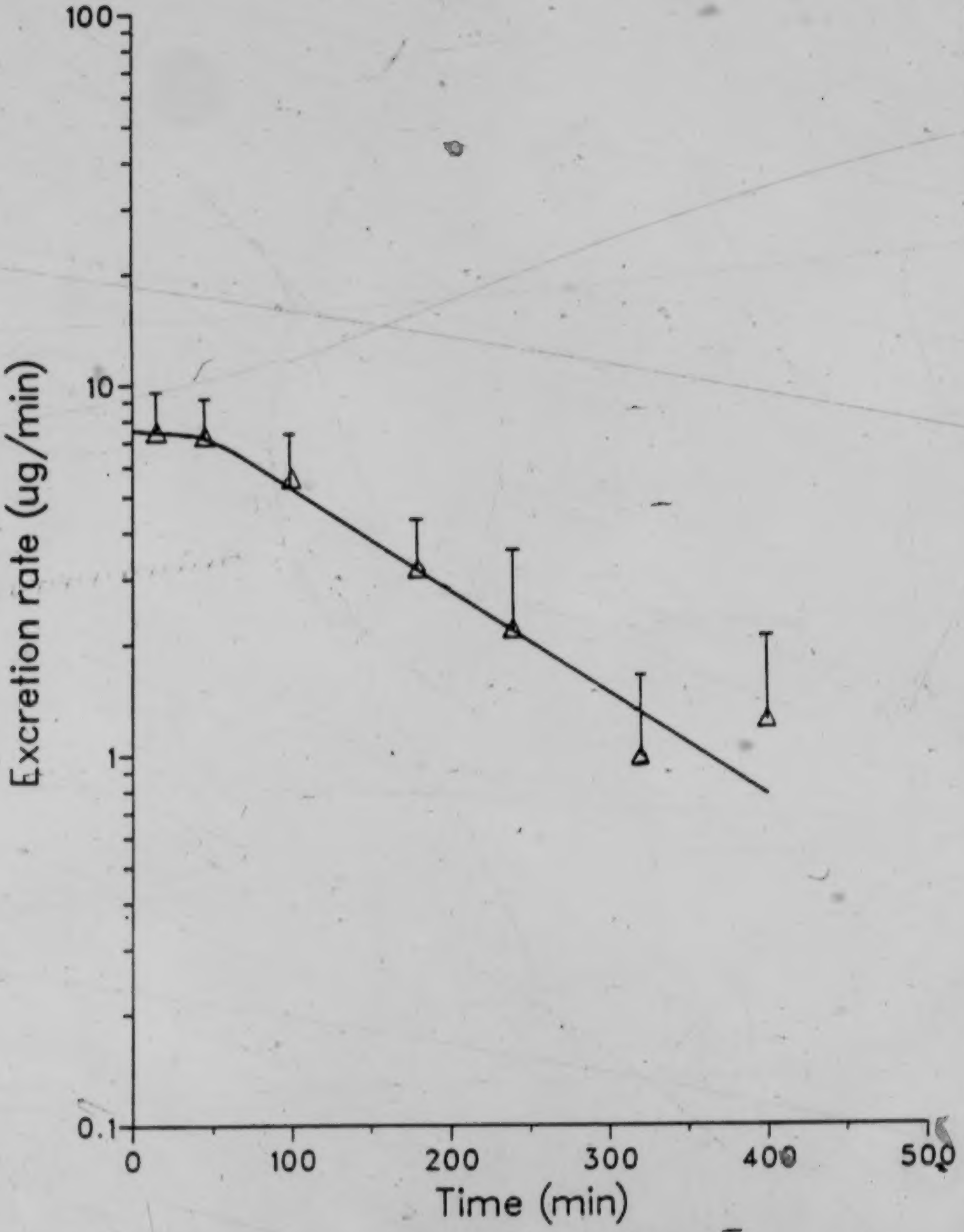


Figure 44. The excretion rate of phenelzine into the bile of rats administered this drug. The bile samples were collected at several time intervals, over a 7 h period, following drug administration.
(Mean ± S.D, N=6)

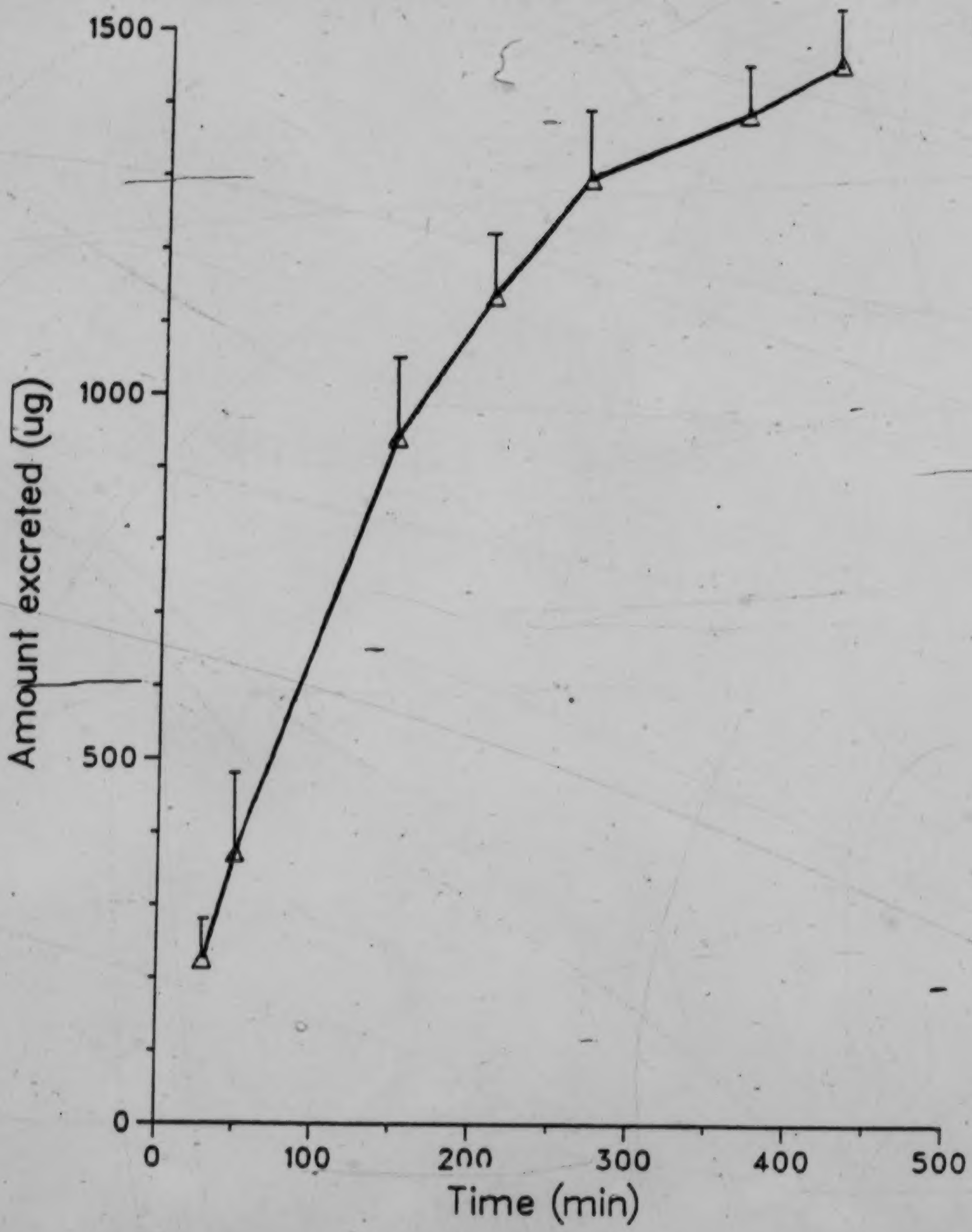


Figure 45. The cumulative excretion of phenelzine into the bile of rats given this drug. The bile samples were collected over a 7 h period following drug administration. (Mean ± S.D., N=6).

Previous studies have looked for this metabolite in blood and urine but no one has examined bile for N'-acetylphenelzine.

Enterohepatic Recirculation

The significant excretion of PLZ into bile, the inability to account for almost 40% of a single i.p. dose of PLZ and the lack of difference between the AUC of sham-operated and bile duct-cannulated rats implies that PLZ does not undergo significant enterohepatic recirculation. However, it is difficult to demonstrate enterohepatic recirculation of a drug which is administered i.v. Differences in the AUC between bile duct-cannulated and sham-operated animals are only observed during the drugs distribution phase when the blood concentration of the drug is high and significant biliary excretion is occurring.

3.1.4.8 Physiological Effects of Chronic Phenelzine Dosing

Chronic i.p. administration of PLZ to rats influenced the physiological status of these animals. During a 21 day experimental period, where animals received daily doses of PLZ (15 mg/kg, free base) a loss of 6 ± 4 g in total body weight was observed. Control animals, treated over the same time period with saline gained 140 ± 10 g (Fig 46). The weight loss observed in PLZ-treated animals could be explained by a loss of appetite or by poor food utilization associated with a decrease in the level of vitamin B6 (334). It has been suggested that PLZ administration causes a reduction in the level of vitamin B6, *in vivo* (168).

The inability of these animals to gain weight was paralleled by a decrease in the weight of certain organs compared to those obtained from age-matched saline treated controls. The liver, lung and kidney of PLZ-treated animals were significantly reduced in weight compared to those of saline treated animals after a 15 day treatment period (Fig 47). The weights of the remaining organs (spleen, heart and brain) were not significantly different from the corresponding organs obtained from the saline-treated controls. It is

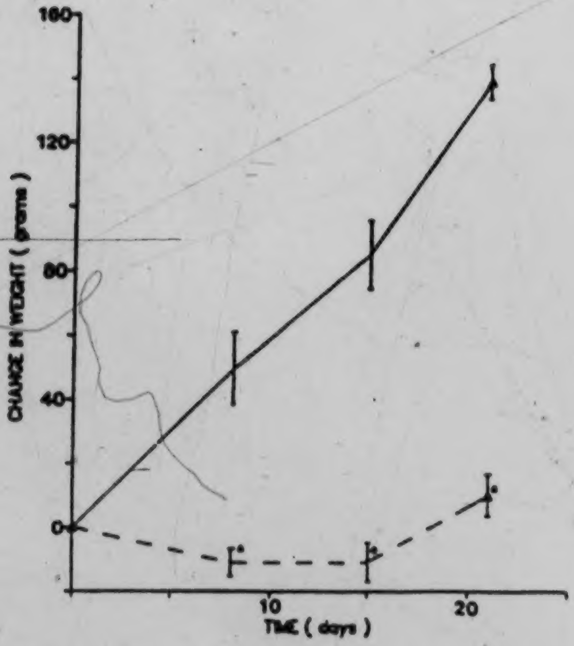


Figure 46. The effect of chronic phenelzine treatment on the total body weight of the rat, compared to saline treated control animals.
(Mean±S.D., N=4)
Saline treated animals————
Multiple doses of Phenelzine ----------
a-significantly different ($P < 0.05$).

interesting that all three organs affected by PLZ treatment are involved in some aspect of drug elimination. The liver, lung and kidney are capable of carrying out CYT P450-mediated oxidations of xenobiotics (334). In addition all three organs appear to be involved in the excretion of PLZ. It is possible that during the conversion of PLZ to EBZ or hydrazine in these organs, damage to the liver, kidney and lung could occur. With chronic dosing, the increased formation of EBZ might lead to even further tissue damage.

3.1.4.9 Conclusion

The disposition of PLZ in the body after single or multiple doses of this drug has never been appropriately evaluated. In this study investigations into the metabolism and pharmacokinetics of PLZ were initiated. The study was designed to determine the influence of chronic PLZ administration on its own metabolism. The use of an animal model, rather than humans, allowed for the collection of a variety of biological fluids (i.e. urine, blood and bile) which could not be readily obtained from human subjects. In addition much of the previous work investigating the formation of PEA from PLZ was done using a rat model, and therefore for consistency the same animal model was selected.

It is evident from this study that the metabolism of PLZ changed with chronic dosing. The increased formation of neutral and basic metabolites coincided with a decrease in the quantity of metabolite(s) in the unextracted (acid) fraction. One of the neutral metabolites was identified as EBZ. Once formed, EBZ was eliminated in the expired air of rats treated with PLZ. Although this metabolite could be isolated and identified in the expired air of these animals, the quantity eliminated by this route could not be determined. In order to quantitate this metabolite accurately in expired air, a method which can be used to trap all the expired EBZ is required. However, regardless of the quantity, the observation that this metabolite is formed has important toxic implications, since EBZ is believed to be derived from PLZ through a free radical process. Free radicals can readily react with tissue components, causing tissue damage.

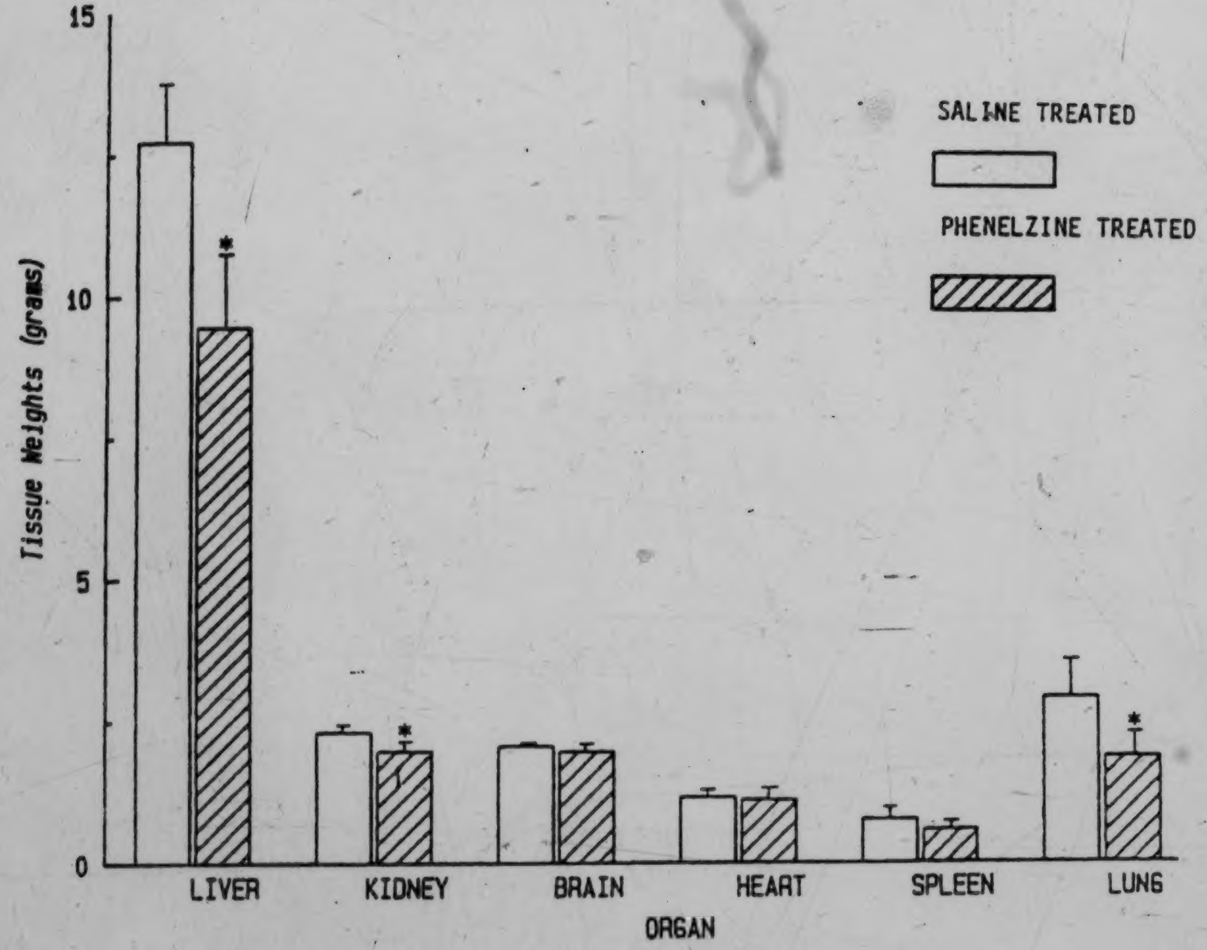


Figure 47. The weights of selected organs following chronic administration of phenelzine sulfate (15 mg/kg, free base). The control animals were treated with saline for the same length of time as phenelzine treated animals. (Mean ± S.D>)*-differs from controls ($P < 0.05$).

PEA is a metabolite of PLZ which possesses pharmacological activity. The delayed increase in PEA levels, observed with chronic PLZ administration, is an unusual response to a MAO B inhibitor. In this study it was found that PEA was formed after an acute dose of PLZ (15 mg/kg). However, it was rapidly cleared from the blood and could not be detected after 30 min. With chronic administration of PLZ there was a significant increase in the amount of PEA found in blood. This increase in PEA is believed to be due either to its increased formation or to a reduction in its renal excretion following chronic PLZ administration. Preliminary observations have indicated that some of the PLZ-treated rats had kidney damage. The reason for this damage was not determined. To further investigate the influence of PLZ administration on kidney function would require histological and physiological evaluation of kidney ultrastructure and function, not only after chronic i.v. administration of PLZ but also after chronic i.p. administration of this drug. It would be of value to correlate the level of kidney damage to the reduction observed in PEA elimination. Since both pathways of PEA elimination (i.e MAO B and renal excretion) became impaired, little of the PEA formed from PLZ would be eliminated from the body. With this evidence it is believed the delay in the increase of PEA, observed by Baker et al. (16), was due to the time needed for damage to the kidneys to occur. Once the damage had occurred, PEA levels in the body would remain high for a prolonged period of time. This theory is supported by the finding that even with elevated levels of PEA in the CNS the quantity of PEA present in the urine was still low.

Forty per cent of a single dose of PLZ could not be accounted for in urine, expired air and tissue. To locate this portion of the dose, investigations into the biliary excretion of PLZ were initiated. A significant quantity of PLZ was found to be eliminated in the bile. With chronic PLZ administration, almost 60% of the PLZ dose could not be accounted for in urine and expired air. It is conceivable that the biliary excretion of PLZ could increase with chronic administration of this drug. The influence of chronic PLZ administration on the biliary excretion of this compound must be investigated further.

3.1.4.10 Future Considerations

Besides EBZ, other toxic metabolites of PLZ, not identified in this study, are known to exist. Hydrazine is believed to be formed in large quantities from PLZ (332). It would be worthwhile to investigate the formation of hydrazine from PLZ and to determine if this compound has any influence on kidney function.

3.2 Studies Conducted on Lidocaine

3.2.1 Results

3.2.1.1 Analysis of Lidocaine and its Metabolites

Most of the available analytical procedures, developed for the analysis of LID and its amine metabolites in biological samples, are incapable of detecting known phenolic metabolites of LID (4-OH-XYL 8, 3-OH-MEGX and 3-OH-LID) which may also be present. Of those GC procedures capable of detecting these amphoteric metabolites, poor extraction efficiency (115,117,184) or inadequate chromatographic separation limits their effectiveness. HPLC is of little use for the analysis of these compounds since positive identification of the metabolites cannot be readily obtained. Linking an HPLC to a MS could overcome this limitation however this technique is in its infancy and has limited accessibility. In the present study, an interest in the phenolic amine metabolites and other potential amphoteric metabolites of LID required the development of a method for the analysis of these compounds (Table 11). A gas chromatographic procedure which would provide adequate chromatographic separation and allow unequivocal mass spectral identification of these compounds was desired. To enhance the extractibility of these compounds into organic solvents, various derivatizing reagents capable of reacting with these compounds in an aqueous medium (aqueous acylation) were investigated. A list of the derivatizing reagents tested is presented in table 12.

Derivatization with Ethyl Chloroformate

Ethyl chloroformate was used to derivatize 4-OH-Xyl and 3-OH-LID in aqueous samples (Fig. 48). This reagent readily reacted with 4-OH-Xyl, yielding one product, the di-derivatized compound 21 (Fig. 49). However, when 3-OH-LID was derivatized with this reagent, significant hydrolysis of the amide bond resulted, yielding the N,O-diethoxycarbonyl derivative of 3-hydroxyxylidine 22 which was identified by GC/MS (Fig. 50).

Derivatization with Acetic Anhydride

All the metabolites of LID (but not LID itself) reacted with acetic anhydride in an aqueous solution. Unlike derivatization with ethyl chloroformate, no hydrolysis of the amide bond of LID, MEGX, GX or their hydroxylated analogs was observed during derivatization with this reagent. Detection of acetyl derivatives, following their gas chromatographic (GC) separation, was achieved using a FID. Identities of all the derivatives formed were confirmed by their EI mass spectral fragmentation pattern (Table 13). Aqueous acetylation of 4-OH-Xyl yielded two products, the N,O-diacetyl derivative of 4-OH-Xyl 24 and monoacetylated 4-OH-Xyl 25. The position of the acetyl group (on either the N or O) could not be determined from the mass spectrum of the latter compound.

Following their extraction, further derivatization of these acetylated compounds with the perfluoroacylating reagent TFAA was investigated. The acetyl ester formed during aqueous acetylation required prior hydrolysis, with 10N ammonium hydroxide, before this compound could be further derivatized. The freed phenolic group can then be further derivatized with TFAA to the trifluoroacetyl ester. Derivatization of 3-OH-LID and 3-OH-MEGX by this procedure (Fig. 51) yielded the same product identified by MS as N,O-bis-trifluoroacetyl-3-OH-Xyl 30 (Fig 52). Hydrolysis of the amide bond of 3-OH-LID was also achieved by treating this compound with TFAA alone, again producing N,O-bis-trifluoroacetyl-3-OH-Xyl (Fig

Table 11. Possible phenolic amine metabolites of lidocaine in man and/or animals. Also included are those phenolic amine metabolites previously identified.

4-hydroxylidocaine (4-OH-LID)
4-hydroxymonoethylglycinexylidide (4-OH-MEGX)
4-hydroxyglycinexylidide (4-OH-GX)
4-hydroxy-2,6-dimethylaniline (4-OH-Xyl)*
3-hydroxylidocaine (3-OH-LID)*
3-hydroxymonoethylglycinexylidide (3-OH-MEGX)*

* previously identified

Table 12. Aqueous derivatizing reagents tried for the derivatization of the phenolic amine metabolites of lidocaine in an aqueous solution.

Reagent	Compounds tested
Ethyl chloroformate	4-hydroxy-2,6-dimethylaniline (4-OH-2,6-xylidine) 3-hydroxylidocaine
Acetic anhydride	monoethylglycinexylidide (MEGX) glycinexylidide (GX) 2,6-dimethylaniline (Xyl) 4-hydroxylidocaine 3-hydroxylidocaine 4-hydroxymonoethylglycinexylidide 3-hydroxymonoethylglycinexylidide 4-hydroxyglycinexylidide 4-hydroxy-2,6-dimethylaniline
Pentafluorobenzoyl chloride	monoethylglycinexylidide glycinexylidide 4-hydroxylidocaine 3-hydroxylidocaine 4-hydroxymonoethylglycinexylidide 3-hydroxymonoethylglycinexylidide 4-hydroxyglycinexylidide 4-hydroxy-2,6-dimethylaniline 2,6-dimethylaniline

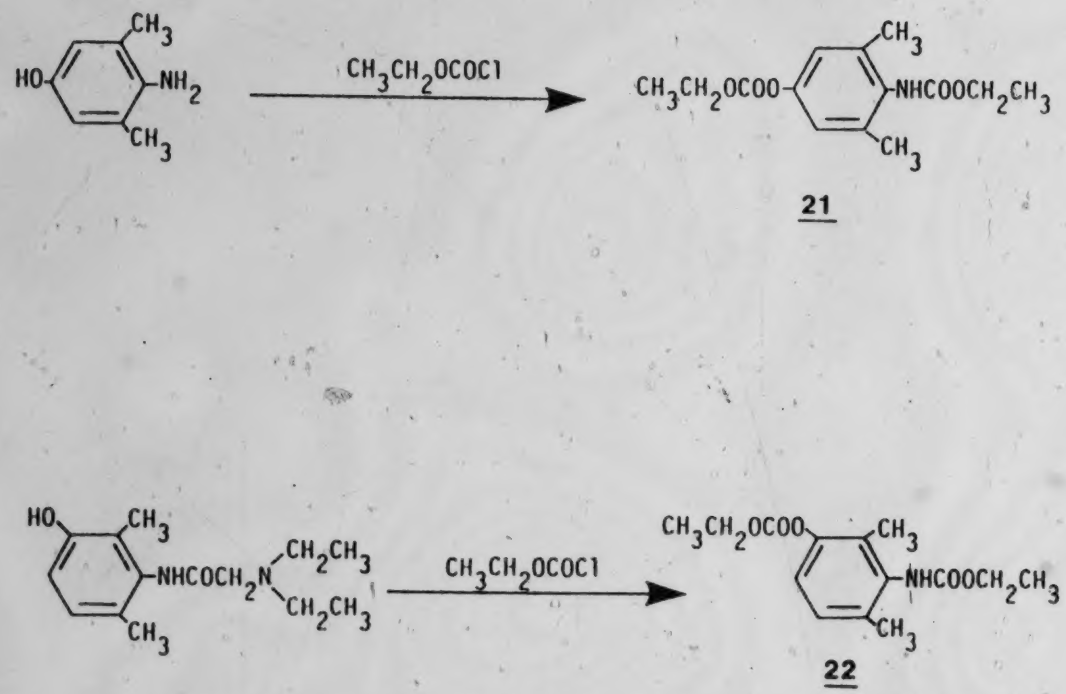


Figure 48. The derivatization of 4-hydroxy-2,6-xylidine and 3-hydroxylidocaine with ethyl choroformate. Products formed were identified by their electron-impact fragmentation patterns.

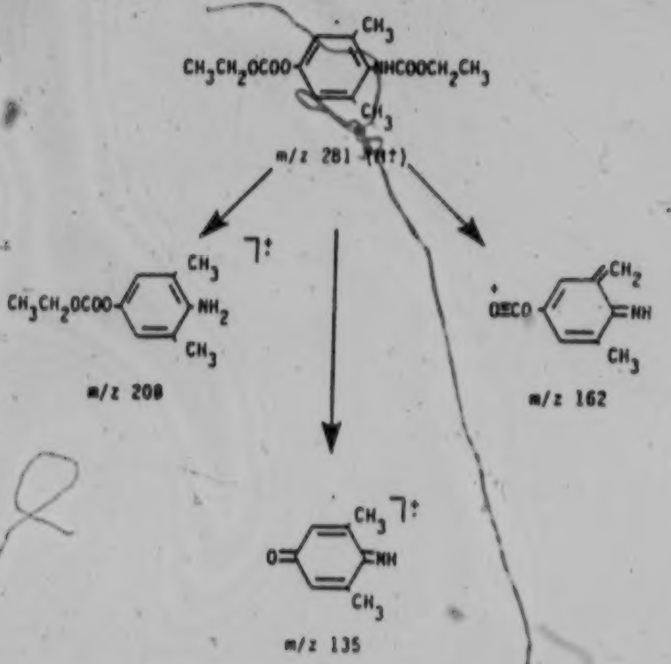
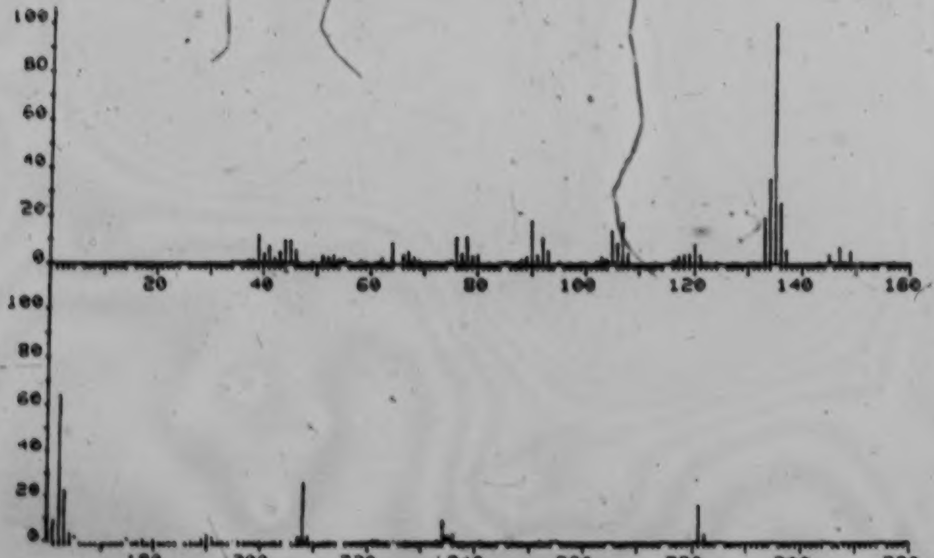


Figure 49. The electron impact fragmentation pattern of 4-hydroxy-2,6-xylidine derivatized with ethyl chloroformate, forming the diderivatized product. The actual mass spectrum of this compound is depicted below.



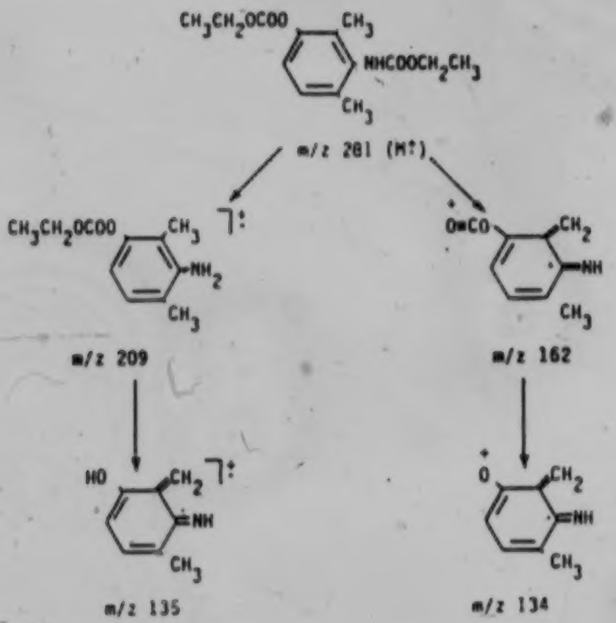


Figure 50. The electron impact fragmentation pattern of the product isolated following the derivatization of 3-hydroxylidocaine with ethyl chloroformate. The actual mass spectrum of this compound is depicted below.

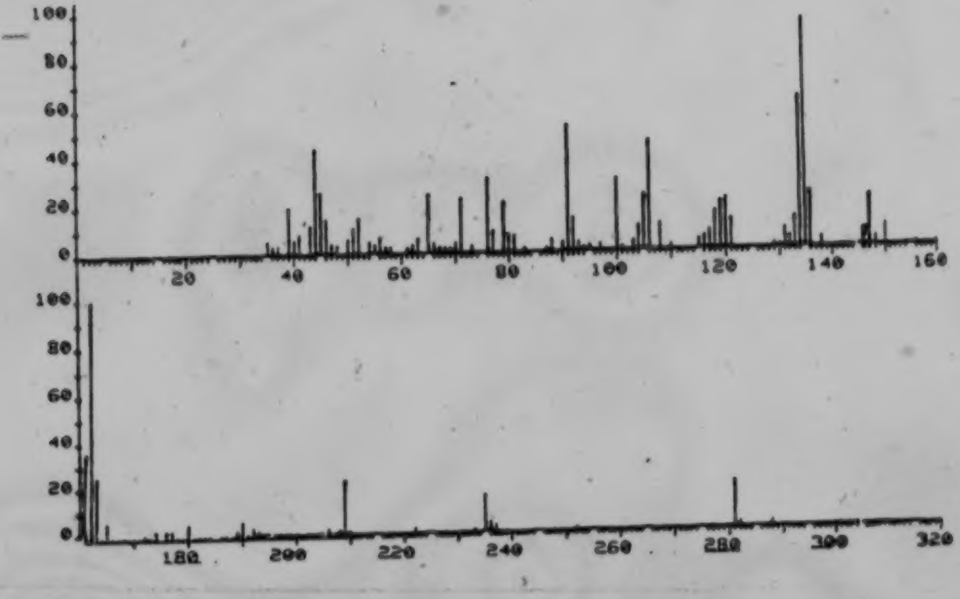
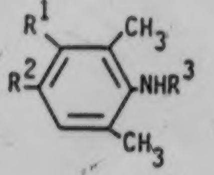


Table 13. Electron-impact mass spectral data of several authentic lidocaine metabolites, derivatized with acetic anhydride.



	R^1	R^2	R^3
23	H	$-OCOCH_3$	$-COCH_2N(COCH_3)CH_2CH_3$
24	$-OCOCH_3$	H	$-COCH_2N(COCH_3)CH_2CH_3$
25	H	$-OCOCH_3$	$-COCH_3$
26	H	$-OH$	$-COCH_3$
27	$-OCOCH_3$	H	$-COCH_2N(CH_2CH_3)_2$
28	H	$-OCOCH_3$	$-COCH_2N(CH_2CH_3)_2$
29	H	H	$-COCH_3$

Generated mass spectra: m/z (% rel. abundance) (structure).

23 $306(4)(M^{+\cdot})$; $179(29)(CH_3COOC_6H_3(CH_3)_2NH_2^{+\cdot})$; $137(33)(HOC_6H_3(CH_3)_2NH_2^{+\cdot})$; $128(100)(\overset{+}{C}OCH_2N(COCH_3)CH_2CH_3)$; $100(68)(\overset{+}{C}H_2N(COCH_3)CH_2CH_3)$; $58(75)(\overset{+}{C}H_2NHCH_2CH_3)$.

24 $306(-)(M^{+\cdot})$; $179(8)(CH_3COOC_6H_3(CH_3)_2NH_2^{+\cdot})$; $137(18)(HOC_6H_3(CH_3)_2NH_2^{+\cdot})$; $128(100)(\overset{+}{C}OCH_2N(COCH_3)CH_2CH_3)$; $100(58)(\overset{+}{C}H_2N(COCH_3)CH_2CH_3)$; $58(73)(\overset{+}{C}H_2NHCH_2CH_3)$.

25 $221(12)(M^{+\cdot})$; $179(35)(HOC_6H_3(CH_3)_2NHCOCH_3^{+\cdot}$ or $CH_3COOC_6H_3(CH_3)_2NH_2^{+\cdot})$; $137(100)(HOC_6H_3NH_2^{+\cdot})$; $136(22)(HOC_6H_3\overset{+}{N}H$ or $\overset{+}{O}C_6H_3NH_2)$.

26 $179(19)(M^{+\cdot})$; $137(100)(HOC_6H_3(CH_3)_2NH_2^{+\cdot})$; $136(29)(\overset{+}{O}C_6H_3(CH_3)_2NH_2$ or $HOC_6H_3(CH_3)_2\overset{+}{N}H)$

27 $292(3)(M^{+\cdot})$; $86(100)(\overset{+}{C}H_2N(CH_2CH_3)_2)$; $58(11)(\overset{+}{C}H_2NHCH_2CH_3)$

Table 13. Continued.

28	292(2)($M^{+\cdot}$); 86(100)($\overset{+}{C}H_2N(CH_2CH_3)_2$); 58(9)($\overset{+}{C}H_2NHCH_2CH_3$).
29	163(54)($M^{+\cdot}$); 121(100)($C_6H_3(CH_3)_2NH_2^{+\cdot}$); 106(85)($121\text{-}15^+$).

53). 3-OH-MEGX, however, was stable to TFAA treatment (Fig 54). The product formed was identified as N,O-bis-trifluoroacetyl-3-OH-MEGX 31 (Fig 55). LID was also hydrolyzed by treatment with TFAA, under anhydrous conditions, to produce N-trifluoroacetyl-2,6-Xyl 32 (Fig 53), identified by its similar EI/MS fragmentation pattern to authentic N-trifluoroacetyl-2,6-Xyl (Fig 56). MEGX, much like 3-OH-MEGX, was stable to treatment with TFAA, forming N-trifluoroacetyl-MEGX 33 (Fig 57). For comparison, 4-OH-Xyl and Xyl were derivatized by this procedure (aqueous acetylation/TFAA) (Fig. 58).

Derivatization with Pentafluorobenzoyl Chloride

Derivatization of all the metabolites of LID by aqueous pentafluorobenzoylation was achieved using the reagent PFBC. All derivatives were identified by their EI/MS fragmentation patterns (Table 14). The derivatives possessed good GC characteristics and were particularly responsive to an ECD. Separation of all the compounds, except PFBC-derivatized 4-OH-Xyl and 3-OH-LID, was achieved using a capillary column containing a DB-1 stationary phase (Fig 59). PFBC-derivatized 4-OH-Xyl and 3-OH-LID could be separated on a DB-1701 stationary phase (Fig 60).

3.2.1.2 Metabolism of Lidocaine

Investigations into the metabolism of LID were initiated in both rats (i.v. and i.p. dosed) and patients receiving an i.v. infusion of LID. Besides metabolism, studies were also conducted to determine the stability of various LID metabolites in urine and during their analysis.

Stability of 4-Hydroxy-2,6-Xylidine

4-OH-Xyl was found to be unstable in basified solutions (335), but the decomposition product(s) has (have) never been identified. If left for 24 h in an alkaline solution (pH 8.0) at room temperature, 4-OH-Xyl was completely converted

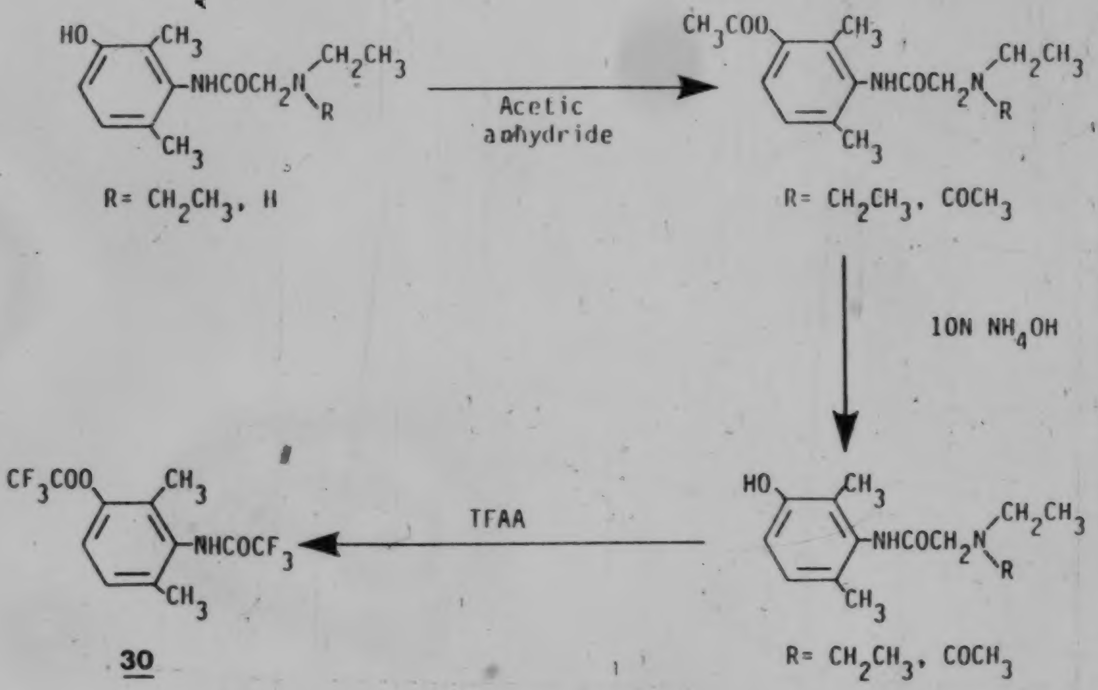


Figure 51. The reaction sequence for the derivatization of 3-OH-MEGX and 3-OH-LID with acetic anhydride followed by trifluoroacetic anhydride. Hydrolysis of the acetyl ester was achieved using 10 N ammonium hydroxide.

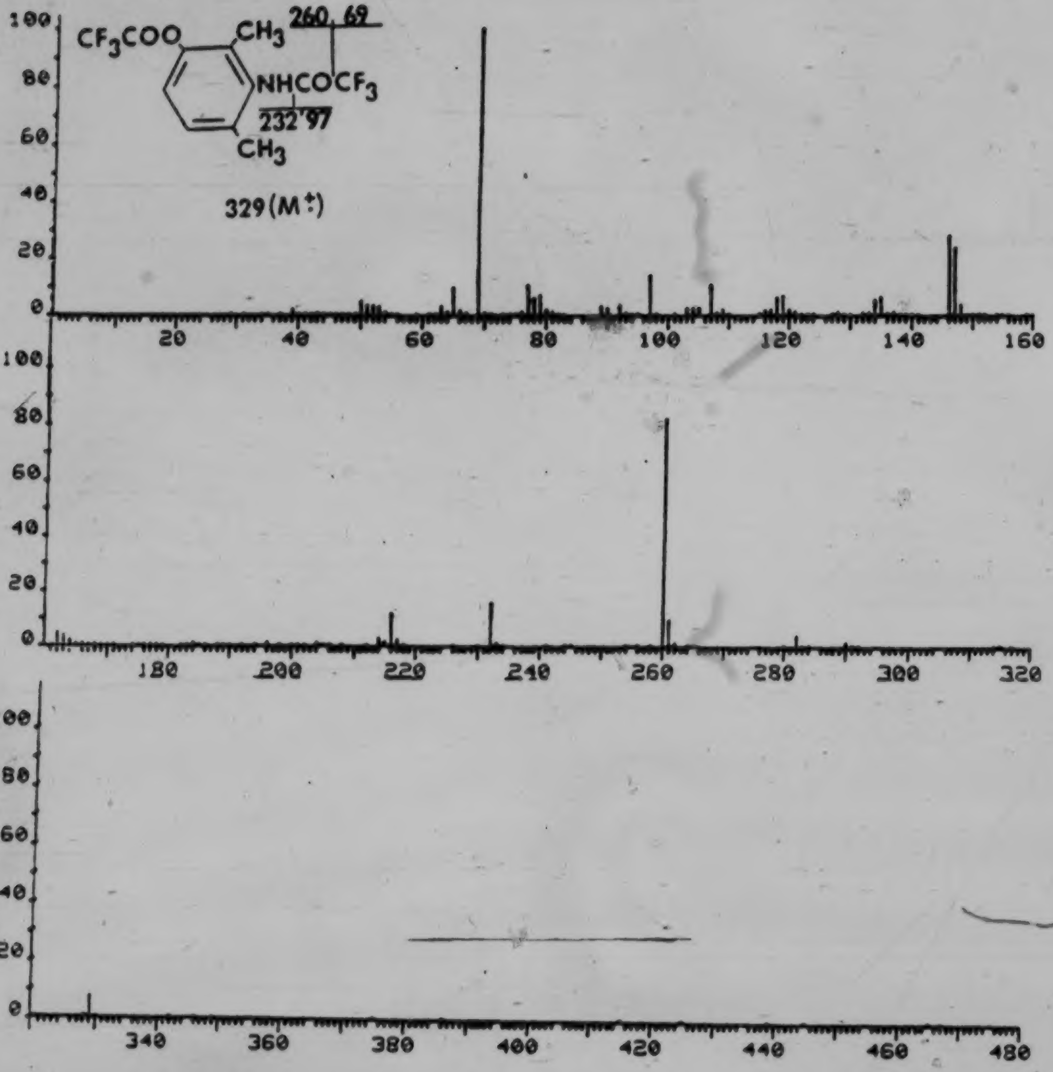


Figure 52. The electron impact mass spectrum of the product formed when 3-hydroxylidocaine was reacted with trifluoroacetic anhydride. The product has been identified as the N,O-ditrifluoroacetyl derivative of 3-hydroxyxylidine. The major diagnostic fragment ions are illustrated above.

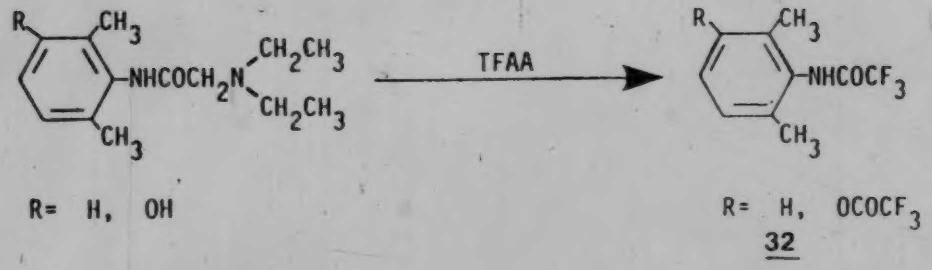


Figure 53. The derivatization of 3-hydroxylidocaine and lidocaine with trifluoroacetic anhydride. The products formed were identified by their electron impact fragmentation patterns.

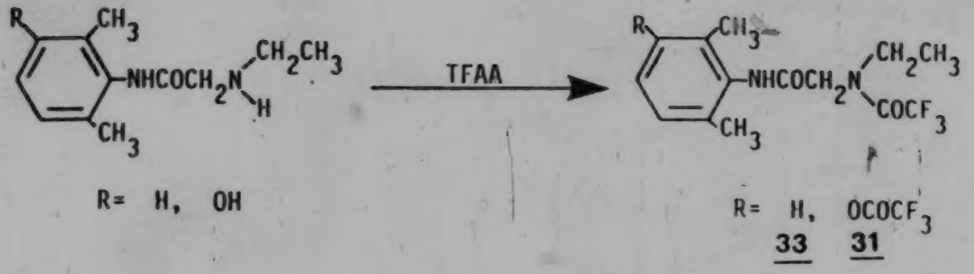


Figure 54. The derivatization of MEGX and 3-hydroxy-MEGX with trifluoroacetic anhydride. The products formed were identified by their electron impact fragmentation patterns.

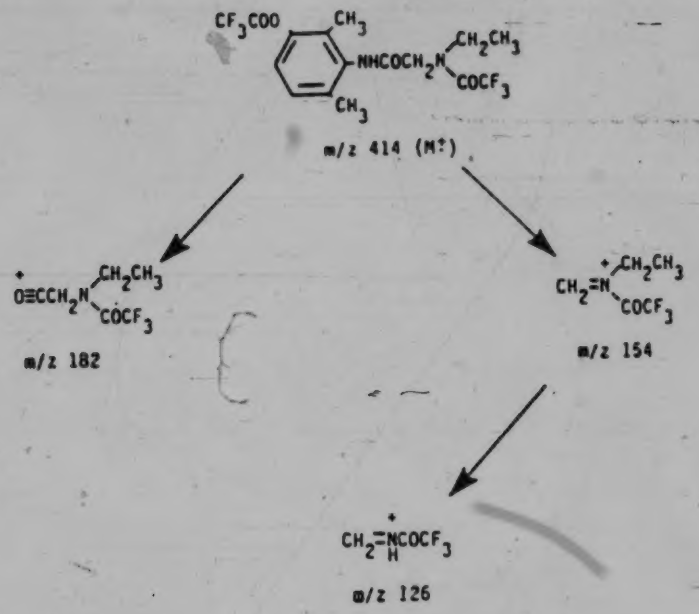
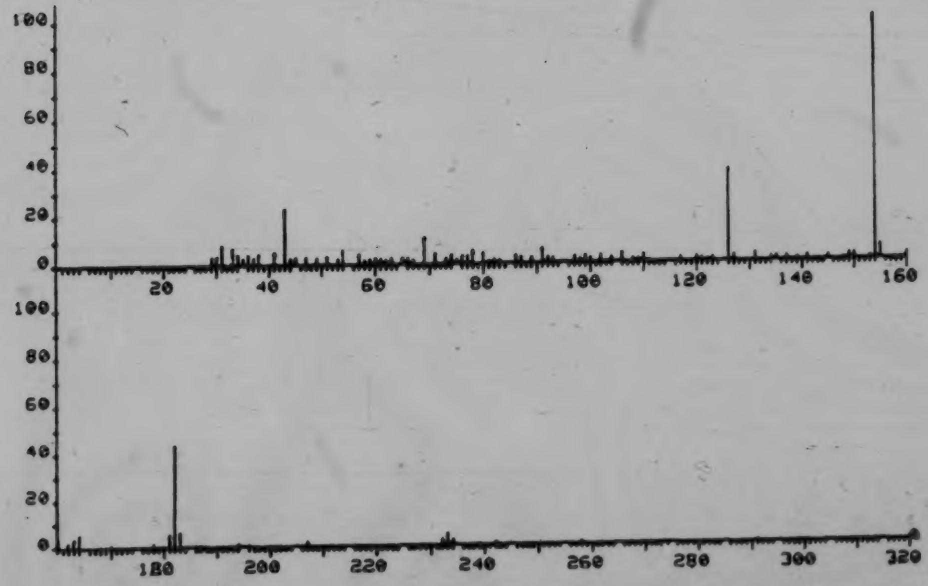


Figure 55. The electron impact fragmentation pattern of N,O-di-trifluoroacetyl-3-hydroxymonoethylglycinexylidide. The actual electron impact mass spectrum of this compound is depicted below.



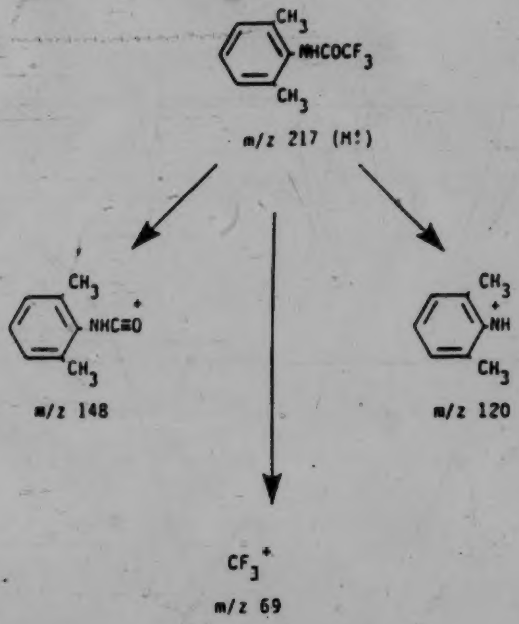
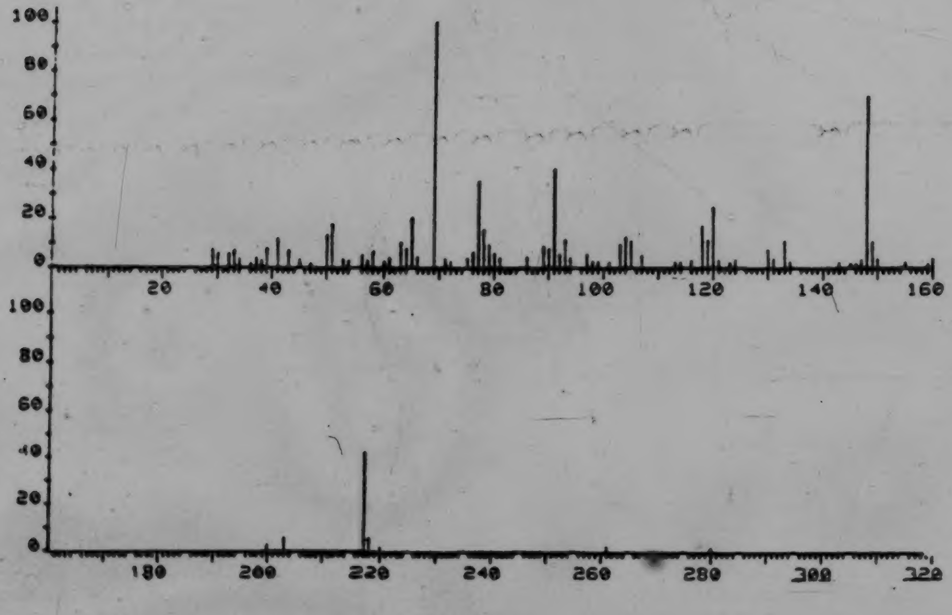


Figure 56. The electron impact fragmentation pattern of N-trifluoroacetyl-2,6-xylidine. The actual electron impact mass spectrum is depicted below.



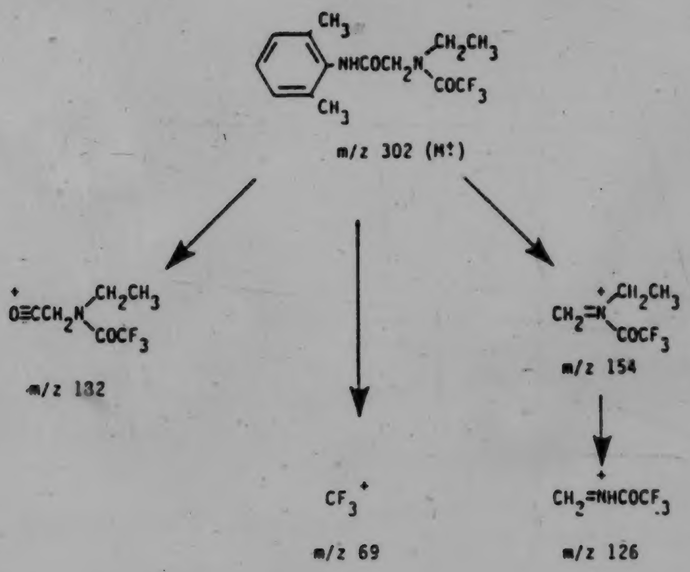
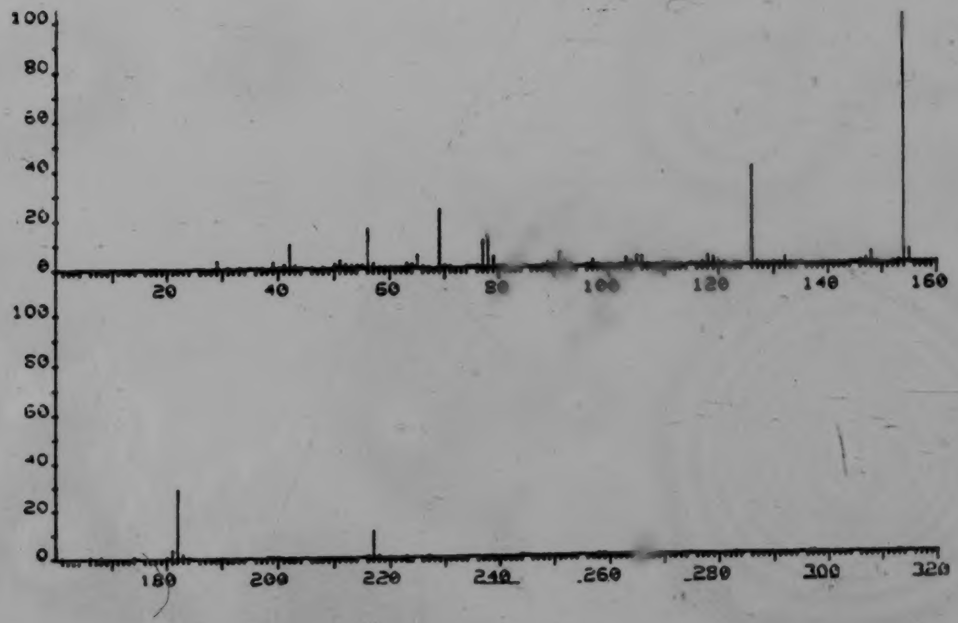


Figure 57. The electron impact fragmentation pattern of N-trifluoroacetylmonoethylglycinexylidide. The actual electron impact mass spectrum is depicted below.



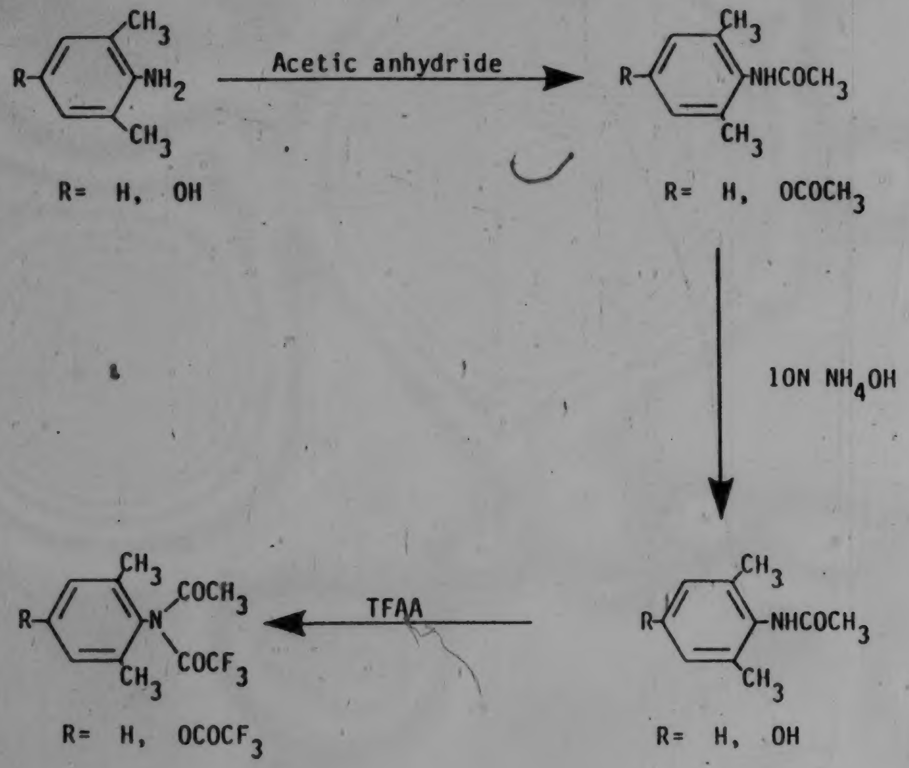
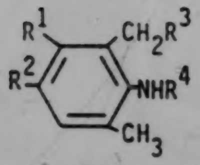


Figure 58. The derivatization of 4-hydroxyxylidine and 2,6-xylidine with acetic anhydride and trifluoroacetic anhydride. Hydrolysis of the acetyl ester of 4-hydroxyxylidine, formed during aqueous acetylation, was achieved with 10N ammonium hydroxide.

Table 14. Electron-impact mass spectral data of several authentic lidocaine metabolites. Prior to analysis these compounds were derivatized with pentafluorobenzoyl chloride.



	R^1	R^2	R^3	R^4
34	H	H	H	$-COCH_2N(CH_2CH_3)COC_6F_5$
35	H	H	H	$-COCH_2NHCOC_6F_5$
36	H	H	H	$-COC_6F_5$
37	H	$-OCOC_6F_5$	H	$-COC_6F_5$
38	H	$-OCOC_6F_5$	H	$-COCH_2N(CH_2CH_3)_2$
39	$-OCOC_6F_5$	H	H	$-COCH_2N(CH_2CH_3)_2$
40	H	$-OCOC_6F_5$	H	$-COCH_2N(CH_2CH_3)COC_6F_5$
41	$-OCOC_6F_5$	H	H	$-COCH_2N(CH_2CH_3)COC_6F_5$
42	H	$-OCOC_6F_5$	H	$-COCH_2NHCOC_6F_5$
New metabolites of lidocaine isolated from the urine of rats given lidocaine.				
43	$-OCOC_6F_5$	H	H	$-COCH_2NHCOC_6F_5$
44 or	$-OCH_3$	$-OCOC_6F_5$	H	$-COCH_2N(CH_2CH_3)COC_6F_5$
45	$-OCOC_6F_5$	H	$-OCH_3$	$-COCH_2N(CH_2CH_3)COC_6F_5$
46	H	H	-OH	$-COCH_2N(CH_2CH_3)COC_6F_5$

Table 14. Continued

Mass spectral data: m/z (% rel abundance) (structure)
Fragment ions common to all spectra are m/z 195 ($^{+}OCC_6F_5$) and m/z ($C_6F_5^{+}$).

34 400(11)($M^{+\cdot}$); 280(24)($\overset{+}{O}CCH_2N(CH_2CH_3)COC_6F_5$); 252(37)($\overset{+}{C}H_2N(CH_2CH_3)COC_6F_5$); 195(100); 167(12); 121(31)($C_6H_3(CH_3)_2NH_2^{+\cdot}$).

35 372(23)($M^{+\cdot}$); 252(2)($\overset{+}{O}CCH_2NHCOC_6F_5$); 224(8)($\overset{+}{C}H_2NHCOC_6F_5$); 195(70); 167(13); 148(48)($C_6H_3(CH_3)_2NHC\overset{+}{O}$); 121(100)($C_6H_3(CH_3)_2NH_2^{+\cdot}$).

36 315(48)($M^{+\cdot}$); 195(48); 167(14); 148(5)($C_6H_3(CH_3)_2NHC\overset{+}{O}$); 120(100)($C_6H_3(CH_3)_2\overset{+}{N}H$).

37 525(19)($M^{+\cdot}$); 330(9)($C_6F_5COOC_6H_3(CH_3)_2\overset{+}{N}H$ or $\overset{+}{O}C_6H_3(CH_3)_2NHCOC_6F_5$); 195(100); 167(16).

38 or 39* 444(2)($M^{+\cdot}$); 357(3)($C_6F_5COOC_6H_3(CH_3)_2NC\overset{+}{O}$); 330(3)($C_6F_5COOC_6H_3(CH_3)_2\overset{+}{N}H$); 195(46); 167(11); 86(100)($\overset{+}{C}H_2N(CH_2CH_3)_2$); 58(51)($\overset{+}{C}H_2NHCH_2CH_3$).

40 or 41* 610(5)($M^{+\cdot}$); 331(12)($C_6F_5COOC_6H_3(CH_3)_2NH_2^{+\cdot}$); 280(28)($\overset{+}{O}CCH_2N(CH_2CH_3)COC_6F_5$); 252(37)($\overset{+}{C}H_2N(CH_2CH_3)COC_6F_5$); 195(100); 167(9).

42 592(19($M^{+\cdot}$); 358(12)($C_6F_5COOC_6H_3(CH_3)_2NHC\overset{+}{O}$); 331(37)($C_6F_5COOC_6H_3(CH_3)_2NH_2^{+}$); 252(6)($\overset{+}{O}CCH_2NHCOC_6F_5$); 224(13)($\overset{+}{C}H_2NHCOC_6F_5$); 195(100); 167(13); 136(48)($OC_6H_3(CH_3)_2\overset{+}{N}H_2$)

New metabolites of lidocaine

43 582(13)($M^{+\cdot}$); 358(78)($C_6F_5COOC_6H_3(CH_3)_2NHC\overset{+}{O}$); 331(75)($C_6F_5COOC_6H_3(CH_3)_2NH_2^{+\cdot}$); 224(20)($\overset{+}{C}H_2NHCOC_6F_5$); 195(100); 167(27). CIMS- 583(87)(MH^{+}).

44 or 45 640(8)($M^{+\cdot}$); 445(3)($CH_3O(C_6F_5COOC_6H_3(CH_3)_2NHCOCH_2\overset{+}{N}HCH_2CH_3$ or C_6F_5COO-$C_6H_3(CH_2OCH_3)(CH_3)NHCOCH_2\overset{+}{N}HCH_2CH_3$); 361(12)($(CH_3O)C_6F_5COOC_6H_3(CH_3)_2$-$NH_2^{+\cdot}$ or $C_6F_5COOC_6H_3(CH_2OCH_3)(CH_3)NH_2^{+\cdot}$); 280(22)($\overset{+}{O}CCH_2N(CH_2CH_3)COC_6F_5$); 252(35)($\overset{+}{C}H_2N(CH_2CH_3)COC_6F_5$); 195(100); 167(5). CIMS- 641(28)(MH^{+}).

46 416(13)($M^{+\cdot}$); 280(15)($\overset{+}{O}CCH_2N(CH_2CH_3)COC_6F_5$); 252(29)($\overset{+}{C}H_2N(CH_2CH_3)COC_6F_5$). 195(100); 167(12); 137(23)($C_6H_3(CH_2OH)(CH_3)NH_2^{+\cdot}$ or $HOC_6H_3(CH_3)_2NH_2^{+\cdot}$).

*- Compounds 39 and 41 are regio-isomers of 38 and 40, respectively. The mass spectra of these compounds are identical to their respective p-isomers except the relative abundances of the fragment ions may be different.

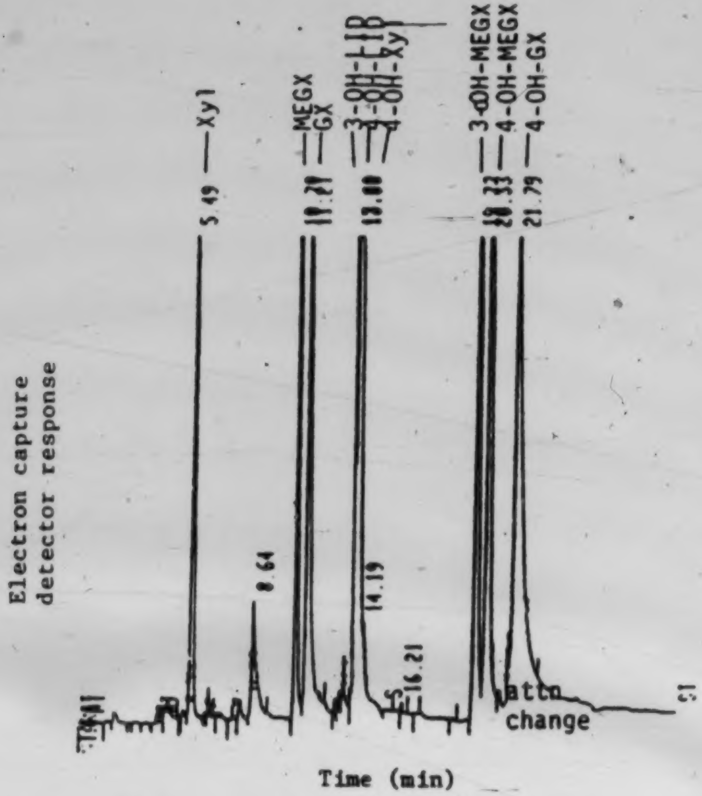


Figure 59. The GC trace of a mixture of Xyl, MEGX, GX, 3-OH-LID, 4-OH-LID, 3-OH-MEGX, 4-OH-MEGX, 4-OH-GX, and 4-OH-Xyl separated on a wall coated open tubular capillary column coated with DB-1 stationary phase. The mixture was derivatized with pentafluorobenzoyl chloride prior to analysis. One microgram of each compound was analysed by this procedure.

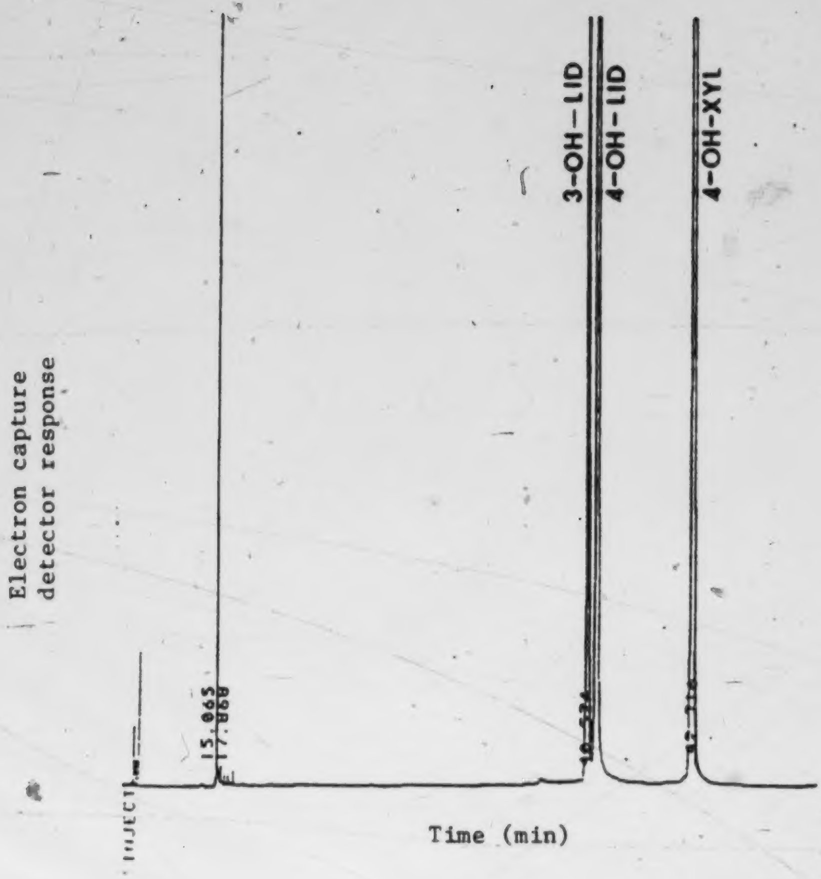


Figure 60. The GC trace of a mixture of 3-OH-LID, 4-OH-LID and 4-OH-Xyl separated on a wall coated open tubular capillary column coated with DB-1701 stationary phase. The mixture was derivatized with pentafluorobenzoyl chloride prior to analysis. The GC run was terminated after 50 min.

to 2,6-dimethyl-p-quinone which was isolated from the aqueous sample by solvent extraction and subsequently identified by its EI/MS fragmentation pattern (Fig 61). A second decomposition product believed to be an intermediate in the breakdown of 4-OH-Xyl to the quinone could also be isolated by dichloromethane extraction of a basified (pH 8.0) freshly prepared aqueous solution of 4-OH-Xyl. This product is believed to be the imino compound, identified by both its chemical ionization (CI) and EI fragmentation pattern (Fig 62,63). For comparison the EI fragmentation pattern of 4-OH-Xyl is depicted in figure 64. The proposed decomposition pathway for 4-OH-Xyl is illustrated in figure 65.

Following acid hydrolysis and enzymatic hydrolysis of glucuronide or sulfate conjugates in urine of patients receiving LID, a significant amount of 4-OH-Xyl could be isolated. When synthetically prepared 4-OH-LID, 4-OH-MEGX and 4-OH-GX, or the 3-OH analogs of LID and MEGX, were subjected to these hydrolytic procedures they were all found to be stable. This suggests that the 4-OH-Xyl, isolated from urine, was not formed during the analysis of other metabolites.

Isolation of the Conjugate of 4-Hydroxyxylidine

To confirm the authenticity of 4-OH-Xyl in urine, the glucuronide conjugate of 4-OH-Xyl was isolated by evaporating to dryness the urine of patients receiving LID. The residue was derivatized with sulfinylmethide carbanion and methyl iodide. Subsequent GC/MS analysis of the permethylated urinary extract demonstrated that 4-OH-Xyl was the only LID metabolite in the urine of these patients as a glucuronide conjugate (Fig 66). Both 3-OH-LID and acetaminophen were used to determine if the aromatic amide bonds were stable to the derivatization procedure. Acetaminophen was readily permethylated, yielding the compound depicted in figure 67. No amide hydrolysis was observed with either compound during derivatization with methyl iodide/sulfinylmethide carbanion. It appears that the 4-OH-Xyl glucuronide conjugate isolated in the urine of these patients was not an artefact formed during the

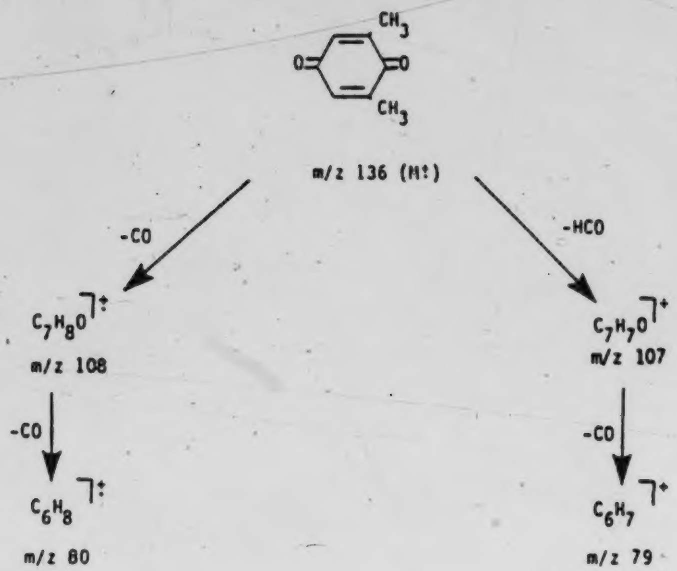
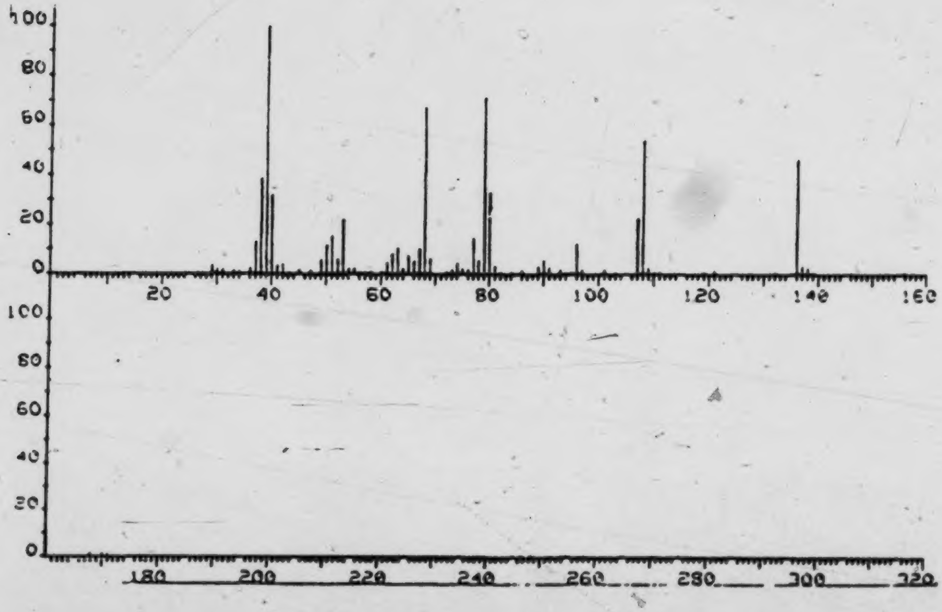


Figure 61. The electron impact fragmentation pattern of the decomposition product of 4-hydroxyxylidine isolated from a basified aqueous solution of 4-hydroxyxylidine left standing at room temperature overnight. The actual electron impact fragmentation pattern of this compound is depicted below.



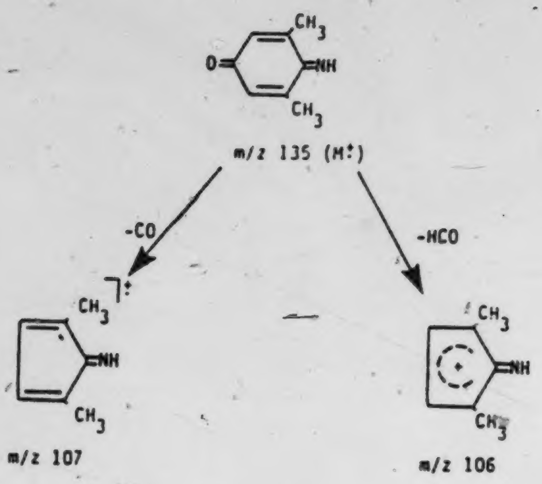
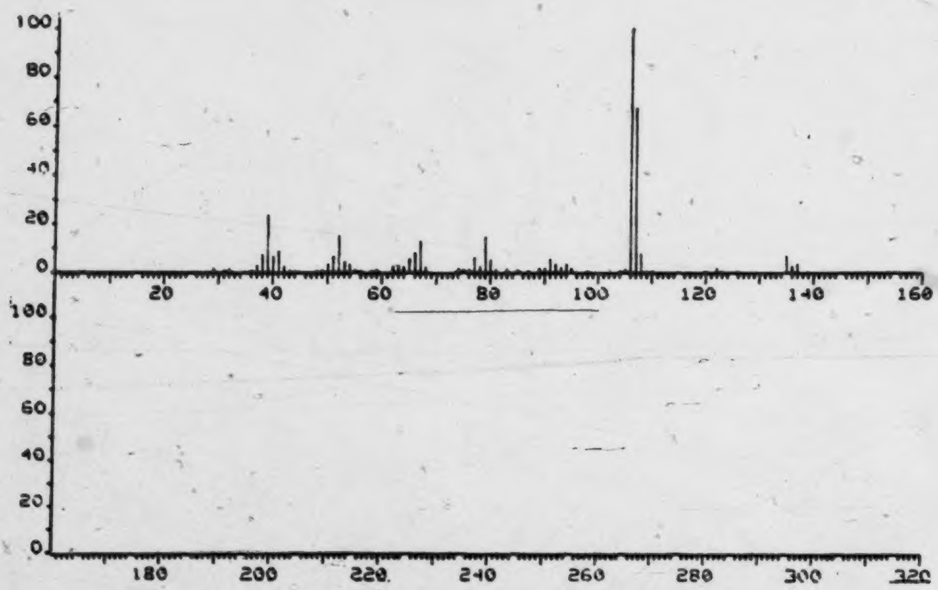
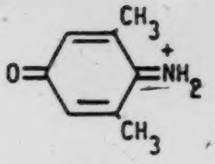


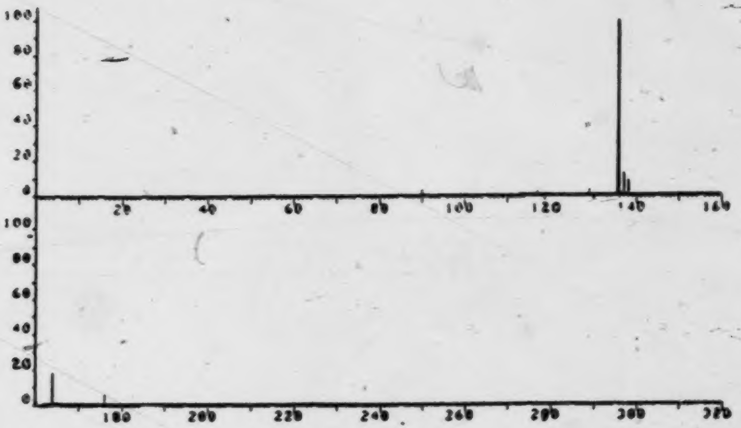
Figure 62. The electron impact fragmentation pattern of the decomposition product isolated, by methylene chloride extraction, from a freshly prepared aqueous solution of 4-hydroxyxylidine. The actual electron impact mass spectrum of this compound is depicted below.





m/z 136 (MH^+)

Figure 63. The structure of the quasimolecular ion identified when the decomposition product of 4-hydroxyxylidine, isolated from a freshly prepared aqueous sample, was subjected to chemical ionization mass spectrometry. The quasimolecular ion was the most abundant ion in the mass spectrum.



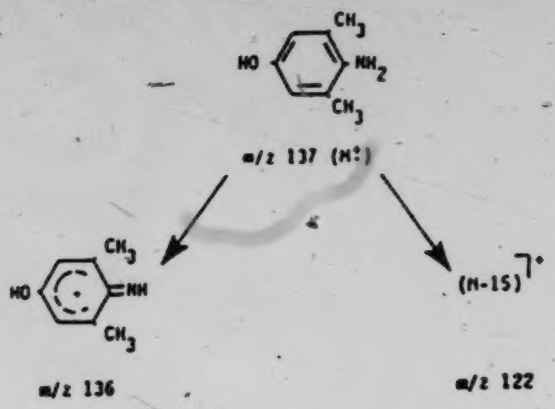
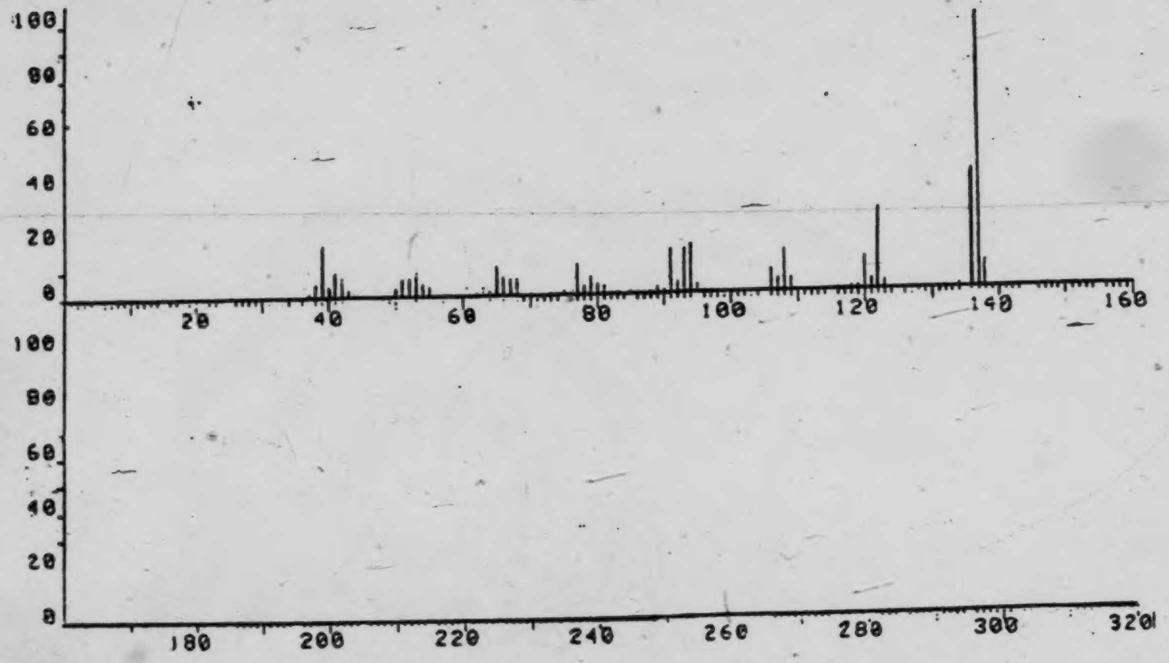


Figure 64. The electron impact fragmentation pattern of 4-hydroxyxylidine, isolated by solvent extraction. The actual electron impact mass spectrum is depicted below.



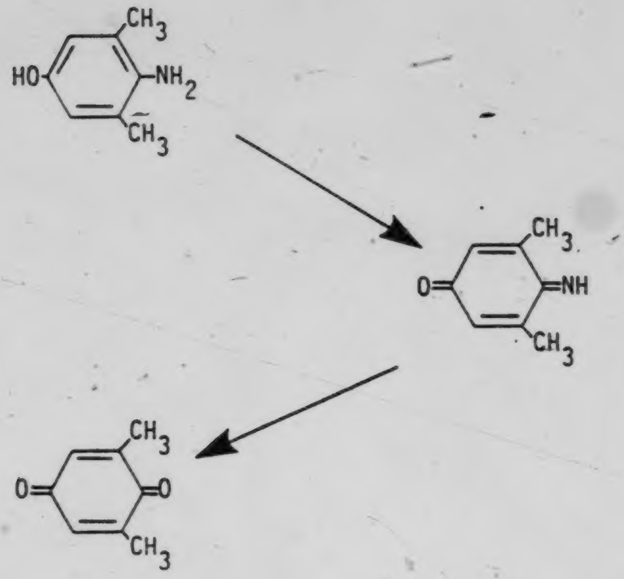


Figure 65. The mechanism proposed for the decomposition of 4-hydroxyxylidine in an aqueous solution adjusted to a basic pH.

derivatization procedure. This procedure was also utilized for the MS analysis of the glucuronide conjugate of acetaminophen, isolated from the urine of rats administered this drug (Fig 68). This was done to confirm that the procedure used was capable of isolating and identifying glucuronide conjugated compounds.

Metabolism of Lidocaine in Rat

LID and the 4-hydroxylated analogs of LID, MEGX and GX were administered individually to rats. Following the administration of 4-OH-GX (50 mg/kg, i.v.) only 4-OH-Xyl could be isolated from the urine of these animals (Fig 69). A similar observation was made following the i.v. injection of 4-OH-MEGX (25 mg, i.v.). The predominant metabolite isolated was again 4-OH-Xyl although some intact parent molecule could also be recovered from these urines (Fig 70). Unlike 4-OH-GX and 4-OH-MEGX, 4-OH-LID could be easily recovered, along with substantial quantities of 4-OH-Xyl from urine following i.v. administration of 4-OH-LID (25 mg, i.v.) to rats. No 4-OH-MEGX was observed in these urines (Fig 71). Both 3-OH-LID and 3-OH-MEGX were isolated from urine following their administration to rats (25 mg, i.v.). In both instances no 3-OH-Xyl was observed. 3-OH-MEGX, however, was identified as a metabolite of 3-OH-LID (Fig 72,73).

Xyl is believed to be the precursor of metabolically produced 4-OH-Xyl (336). When Xyl (30 mg, i.p.) was administered to rats, 4-OH-Xyl was readily isolated from their urine (Fig 74). Also present in the urine was intact Xyl which was estimated to amount to approximately 30% of the isolated urinary metabolites.

Isolation of New Lidocaine Metabolites

When LID was administered to rats, three new metabolites were identified, following derivatization with PFBC, in the urine (Fig. 75). Both CI and EI mass spectra were obtained and used to confirm the identities of these two compounds (Table 14). One of these metabolites was tentatively identified as 3-OH-GX.

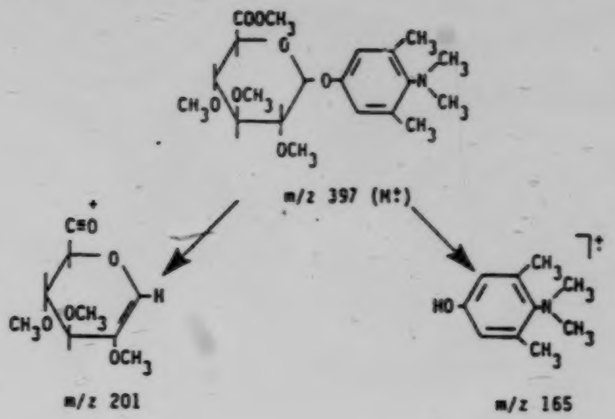
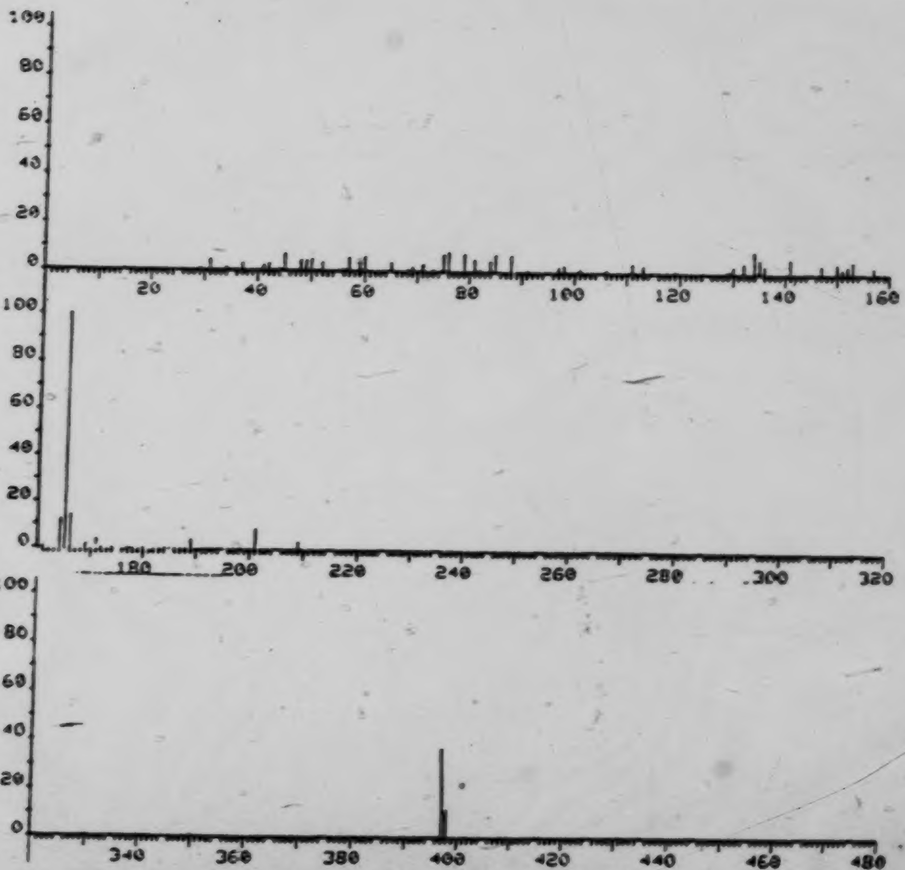


Figure 66. The electron impact fragmentation pattern of the glucuronide conjugate of 4-hydroxyxylidine, permethylated with methyl iodide/dimethylsulfinylmethide carbanion.



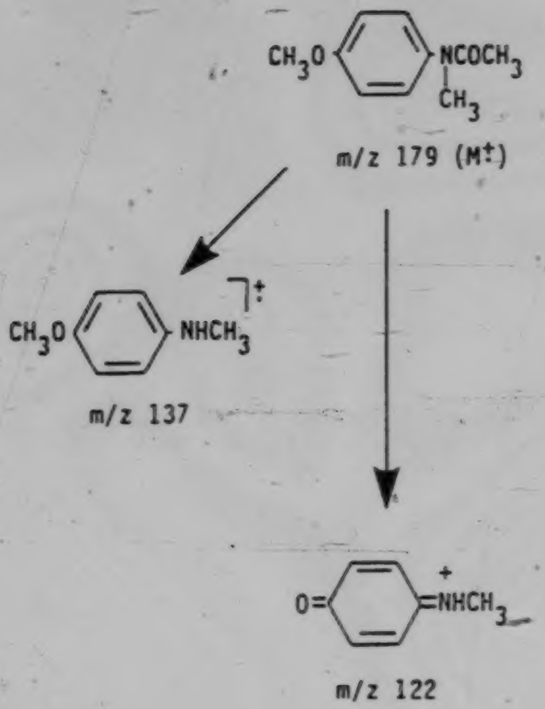
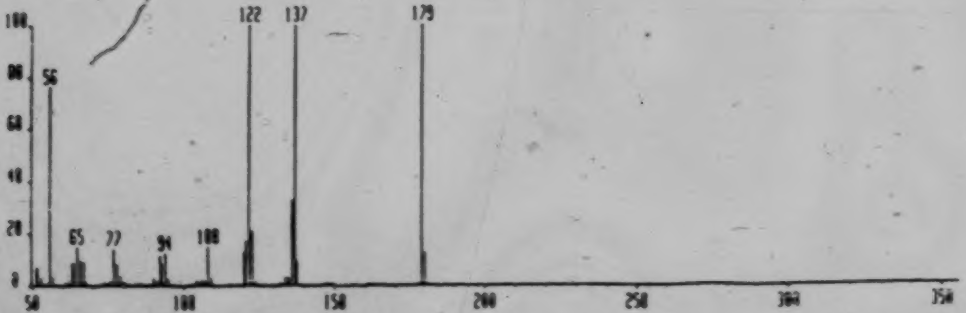


Figure 67. The electron impact fragmentation pattern of acetaminophen permethylated with methyl iodide/dimethylsulfinyl-methide carbanion. The actual electron impact mass spectrum of this compound is depicted below.



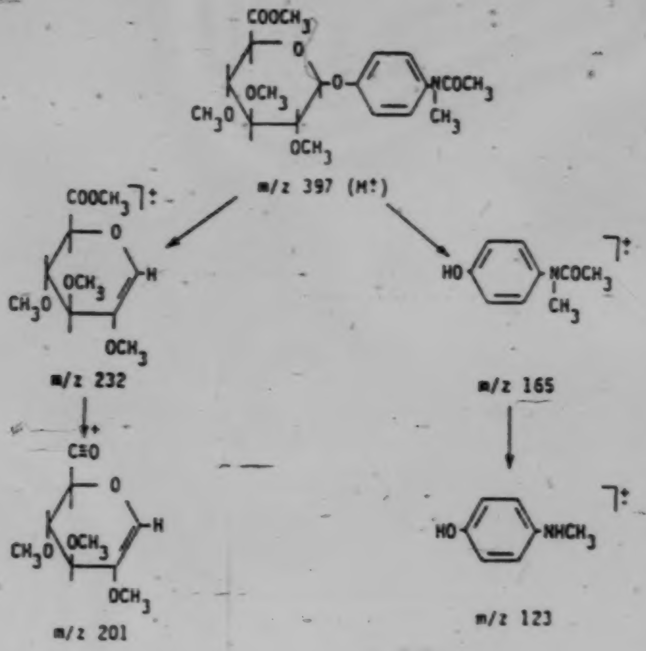


Figure 68. The electron impact fragmentation pattern of the glucuronide conjugate of acetaminophen permethylated with methyl iodide/dimethylsulfinyl-methide carbanion. The actual electron impact mass spectrum of this compound is depicted below.

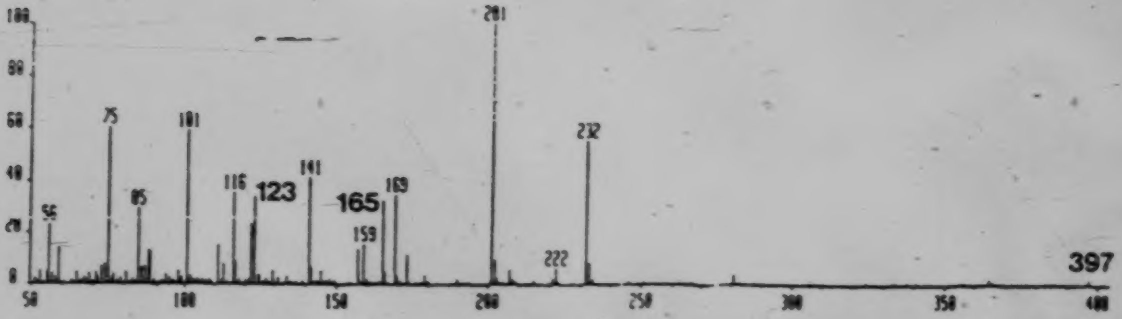


Figure 69. The GC/MS trace of the acetylated extract of urine obtained from rats administered 4-hydroxyglycinexylidide (4-OH-GX) (50 mg, i.v.). The peaks are identified as 1 = monoacetylated derivative of 4-hydroxy-2,6-xylidine; 2 = N,O-diacetylated derivative of 4-hydroxy-2,6-xylidine; 3 = unidentified endogenous constituents or contaminants.

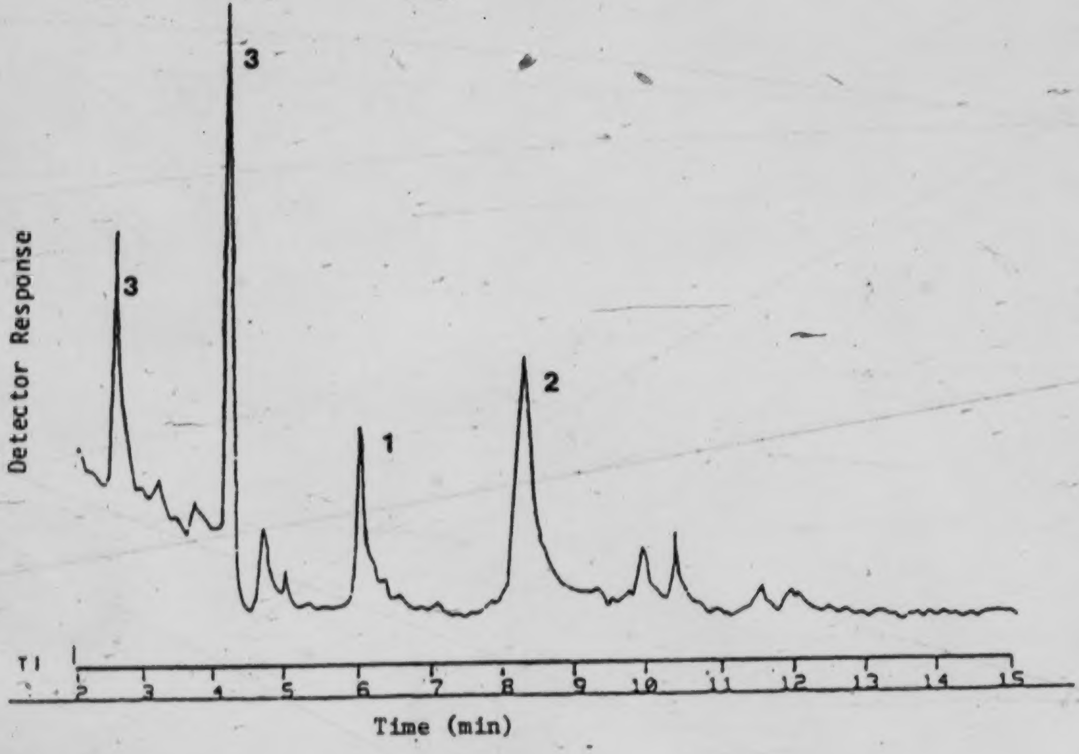
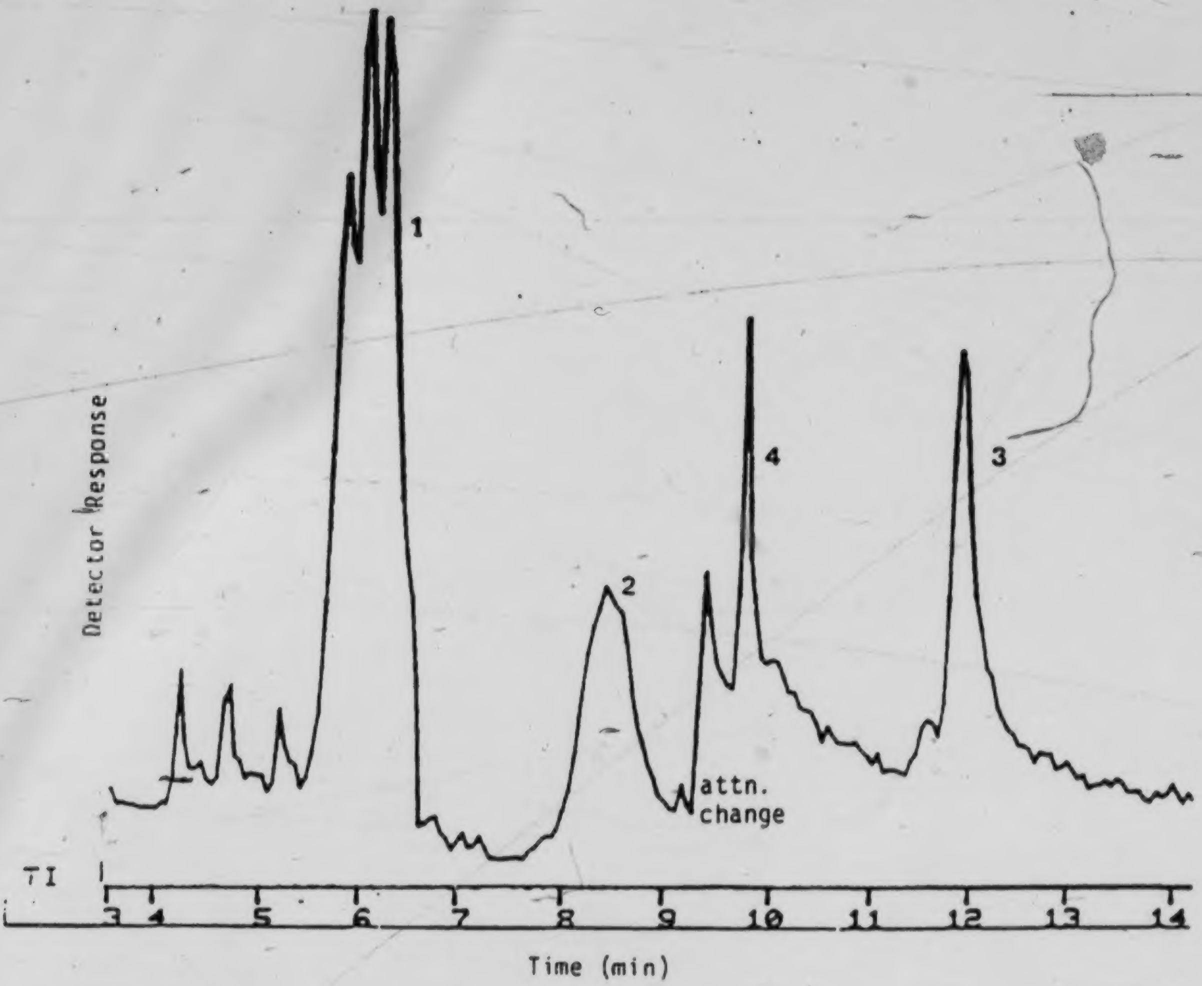


Figure 70. The GC/MS trace of an acetylated extract of urine obtained from rats given a dose of 4-hydroxymonoethylglycinexylidide (4-OH-MEGX) (25 mg, i.v.). The peaks present are identified as 1 = monoacetylated derivative of 4-OH-2,6-xylidine; 2 = N,O-diacetylated derivative of 4-hydroxy-2,6-xylidine; 3 = N,O-diacetylated derivative of 4-hydroxymonoethylglycinexylidide; 4 = unidentified endogenous peaks.



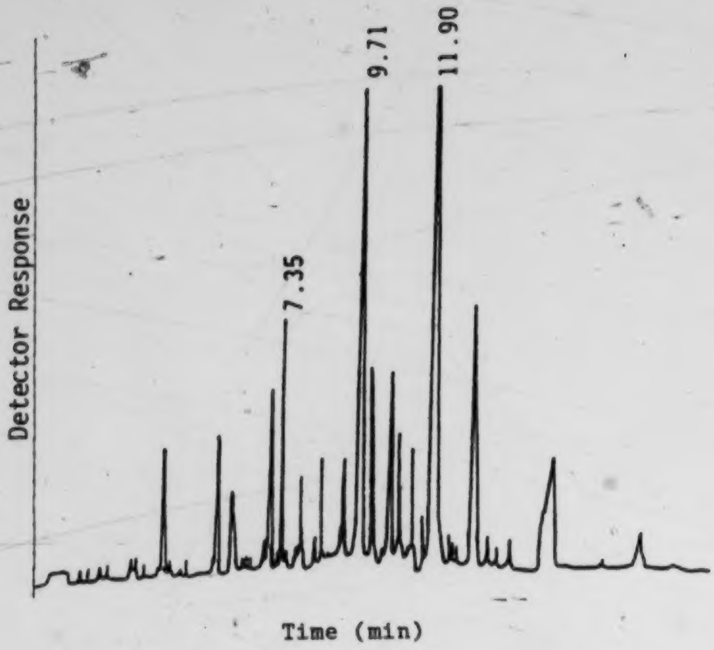


Figure 71. The GC trace of the acetylated extract of urine obtained from rats treated with 4-hydroxylidocaine (4-OH-LID) (25 mg,i.v.). The compounds present are identified by their retention times; 4-hydroxylidocaine (11.90 min) and 4-hydroxyxylidine (7.35 and 9.71) identified as the mono- and di-derivatized compounds, respectively. The other peaks present in the chromatogram were not identified.

Figure 72. The GC/MS trace of the acetylated extract of urine obtained from rats treated with 3-hydroxylidocaine (3-OH-LID) (25 mg, i.v.). The lidocaine metabolites present in the chromatogram are identified as follows; 1=monoacetylated 3-OH-LID and 2=diacetylated 3-OH-MEGX.

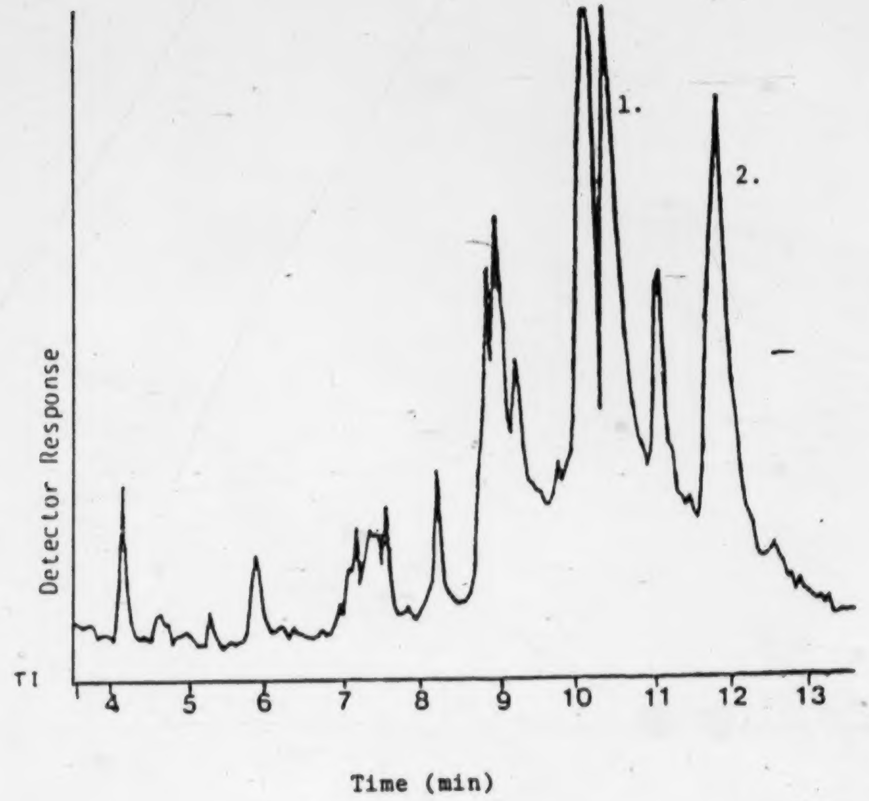
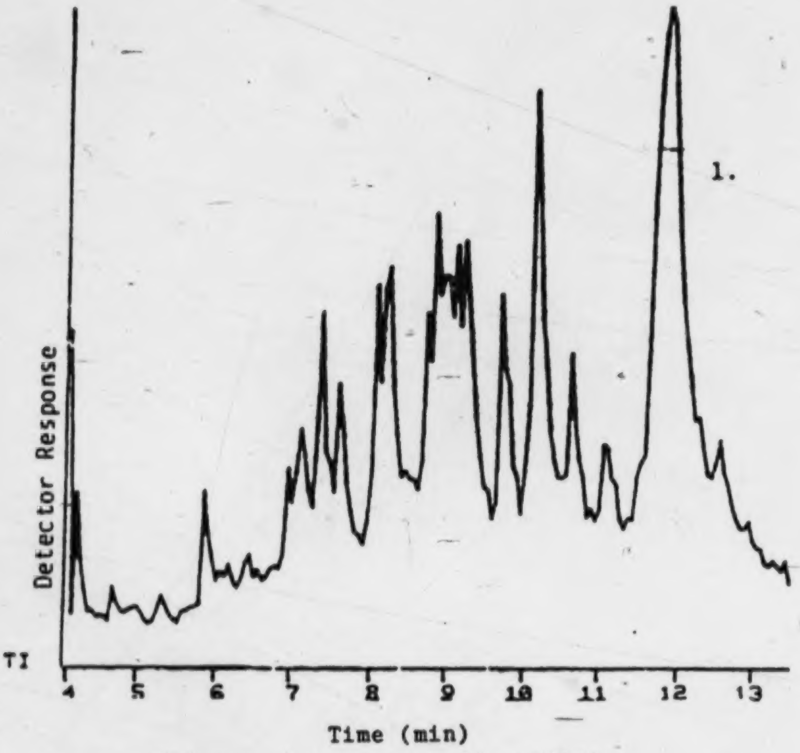


Figure 73. The GC/MS trace of an acetylated extract of urine obtained from rats administered 3-hydroxymonoethylglycinexylidide (3-OH-MEGX) (25 mg, i.v.). The metabolites present in the chromatogram are identified as follows: 1=N,O-diacetylated derivative of 3-OH-MEGX.



- remaining peaks were not identified.

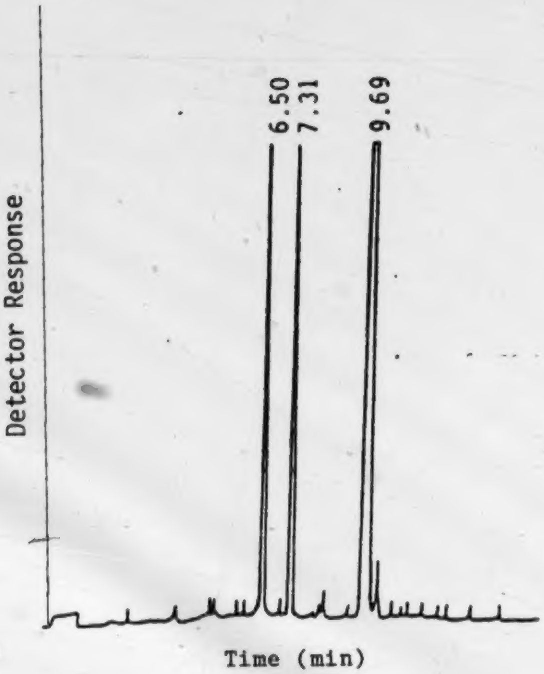


Figure 74 The GC trace of an acetylated extract of rat urine obtained from a rat treated with 2,6-xylidine (40 mg, i.p.). The compounds present in the chromatogram are identified by their retention time: 2,6-xylidine (6.50 min); 4-hydroxyxylidine (7.31, 9.69) as its mono- and di-derivatized product, respectively.

Confirmation of 3-OH-GX was made even though no authentic standard was available, by a) demonstrating the similarity in fragmentation pattern to 4-OH-GX, similarly derivatized, and b) showing the two compounds had different GC retention times (21.59 vs 22.38 min for PFBC-derivatized 3-OH-GX and 4-OH-GX, respectively). The second metabolite was determined to possess one of the two structures illustrated in figure 76. The identification of this compound was based on its EI and CI fragmentation pattern. The molecular ion of this metabolite was found to be 30 amu greater in mass then the molecular ion of 3-OH-MEGX. However, the presence of fragment ions with a mass of either 252 and 280 in the mass spectrum indicated that the aliphatic side chain was the same as PFBC-derivatized 3-OH-MEGX. It is evident from the fragmentation pattern that the structure of the metabolite is similar to 3-OH-MEGX except there is an additional methoxy group attached to the ring. A third metabolite was observed during EI/MS analysis of the PFBC derivatized urinary extract. This metabolite is believed to have the structure 49 depicted in figure 77. The OH group had to be aliphatic since it did not react with PFBC during the derivatization procedure. The aliphatic side chain is again related to MEGX having the characteristic m/z 280 and m/z 252 ions. The mass spectral data for this compound is given in table 14.

The PFBC derivatization procedure was also used for the isolation and identification of 4-OH-MEGX in the urine obtained from a patient treated with LID (Fig 78). In addition 3-OH-MEGX, but not 4-OH-LID and 4-OH-GX, were observed in this urine. The ability to isolate 4-OH-MEGX from this urine was attributed to the development of an improved analytical procedure.

Few studies have been conducted on the phenolic metabolites of LID. This is in part due to the lack of appropriate techniques for their separation and identification. Studies conducted here were directed towards the identification of those metabolites formed as a consequence of metabolism or as artifacts formed during the

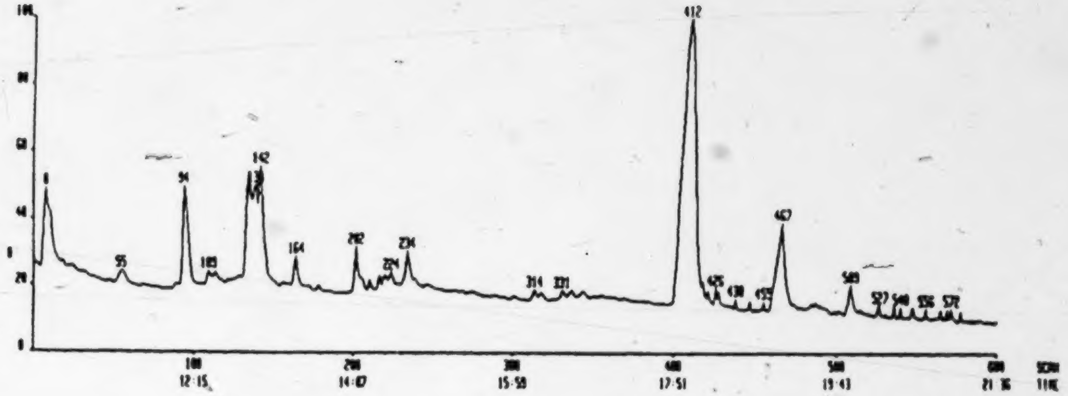
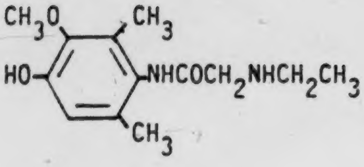


Figure 75. The GC/MS trace of a PFBC-derivatized extract of rat urine obtained from rats treated with lidocaine (40 mg/kg, i.p.). The metabolites present are identified as follows: 138=tentatively identified as the PFBC derivatized benzylalcohol analog of MEGX, 142= PFBC-derivatized 4-OH-Xyl, 412= PFBC-derivatized 3-OH-MEGX, 467= PFBC-derivatized 3-OH-GX and 509= PFBC-derivatized methoxylated analog of 3-OH-MEGX.



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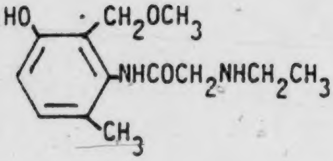
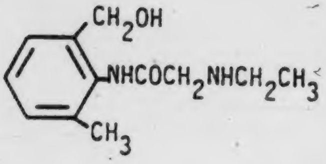


48

Figure 76. The two possible chemical structures proposed for the methoxylated metabolite of lidocaine isolated from the urine of rats, treated with lidocaine (40 mg/kg, i.p.).



49

Figure 77. The proposed structure of the metabolite isolated from the urine of rats administered lidocaine (40 mg/kg, i.p.). This metabolite was isolated from the urine as its pentafluorobenzoylated derivative.

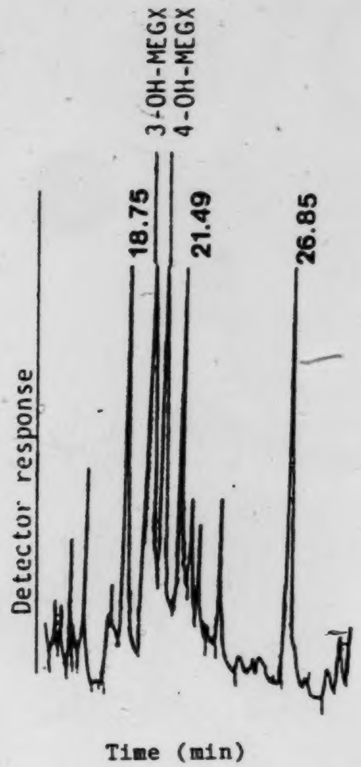


Figure 78. The GC trace of an extract of urine derivatized with pentafluorobenzoyl chloride. The urine was obtained from a patient receiving an i.v. infusion of lidocaine. The retention time of the identified lidocaine metabolites are 3-OH-MEGX (20.04 min) and 4-OH-MEGX (20.62 min). The identities of these two lidocaine metabolites were confirmed by comparing their EI and CI mass spectra with spectra recorded for authentic standards of these compounds similarly derivatized. The remaining peaks present in the GC trace were not identified.

analytical procedure. It is evident that certain analytical procedures could result in the formation of artifacts, particularly during procedures used to hydrolyze glucuronide or sulfate conjugates. The improved method of derivatization developed in this study allowed new metabolites of LID to be discovered, including 4-OH-MEGX in the urine of humans.

3.2.2 Discussion

During prolonged LID infusion, in both rats and man, steady state blood concentrations of LID could not be achieved (48,49,337). Numerous studies have been devoted to investigating this phenomenon. Current theories have suggested that, in man, MEGX accumulates and causes an end-product inhibition of LID de-ethylation (51). More recent studies utilizing an isolated rat liver preparation have indicated, at least in this species, that the hydroxylation pathway rather than the de-ethylation pathway is saturated (52,53). The enzyme responsible for de-ethylation is believed to have a high capacity for LID and is not readily saturated. The metabolic pathway of LID metabolism in the rat (Fig 79) is different from the routes of LID metabolism described for man (Fig 80). Unlike rats which produce 3-OH-LID and 3-OH-MEGX as major metabolites of LID (70% of the dose), in man 4-OH-Xyl is the major metabolite (70% of the dose) (115). Saturation of the hydroxylation pathway in man would not be expected to cause an increase in the blood concentration of LID. Rather significant increases in Xyl would be expected, which was not observed.

The p-hydroxylation of LID, MEGX or GX has never been thoroughly investigated, in part due to the lack of appropriate techniques. However, evidence obtained from several studies suggest these metabolites are possible. Infants administered LID excreted large quantities of LID (20% of the dose) and MEGX (20%) in urine, but, only 1/10 the quantity of 4-OH-Xyl excreted by adults was observed in infants (116). These infants apparently have an impaired ability to hydroxylate Xyl. Using the currently accepted metabolic pathway of LID one would expect a significant increase in the level of Xyl, and not LID or MEGX as is observed, if the

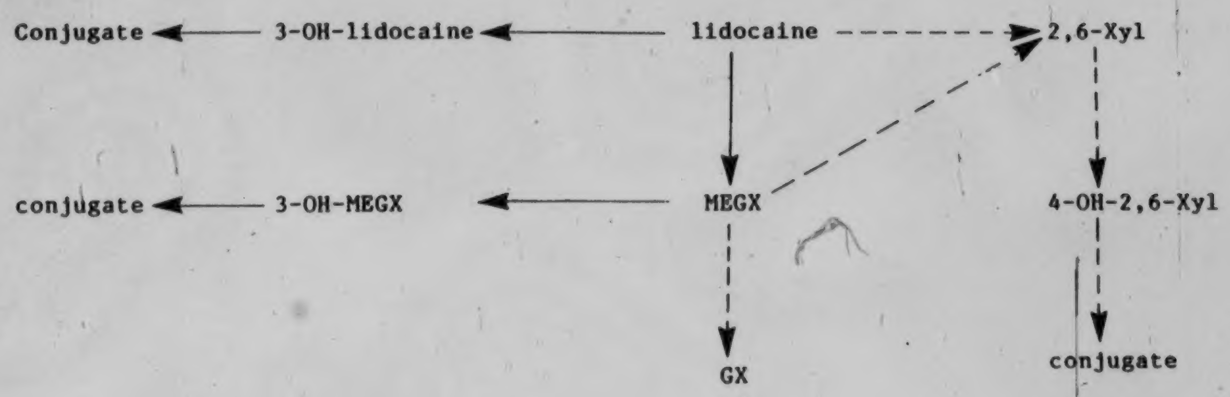


Figure 79. The reported pathway of lidocaine metabolism in the rat. The conjugates formed are either glucuronide oar sulfate conjugates.
Minor pathways -------
Major pathways ———

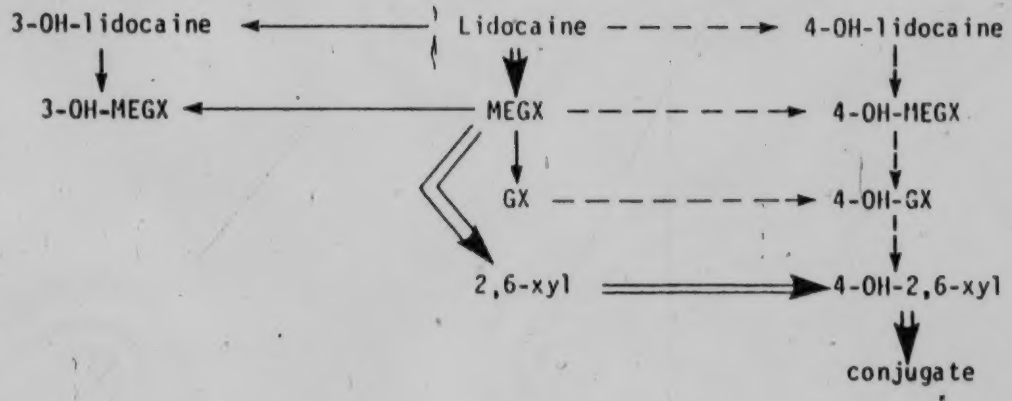


Figure 80. The reported pathways of lidocaine metabolism in man. In addition the metabolites 4-OH-LID, 4-OH-MEGX and 4-OH-GX, are included in the metabolic scheme (-----).
Previously reported pathways ———
Proposed pathways of lidocaine metabolism -----
Major pathways of lidocaine metabolism in man ═══

capacity to p-hydroxylate Xyl was reduced. The level of Xyl accounted for only 2% of the dose of LID, which is similar to the quantity formed in adults.

It was also observed with compounds (mepivicaine, etidocaine and bupivicaine) structurally related to LID demonstrated that man is capable of hydroxylating compounds of this type (338-340). With these compounds hydrolysis of the amide bond did not occur or only accounted for a small percentage of the dose.

Investigations conducted here were initiated to determine if any 4-OH-metabolites (4-OH-LID, 4-OH-MEGX or 4-OH-GX) were present in human urine and, if not, to determine if their absence was due either to chemical or metabolic conversion of these compounds to 4-OH-Xyl, the metabolite normally isolated from human urine of patients treated with LID. Chemical conversion could occur during the hydrolysis procedure (acid or enzymatic), used to convert the conjugated phenolic compounds to their free form. The phenolic metabolites of LID (4-OH-Xyl, 3-OH-LID, 3-OH-MEGX) excreted in urine of rats given LID are present extensively conjugated to glucuronic acid (115). It is assumed that, if present, the 4-hydroxylated analogs of LID, MEGX and GX would also be conjugated to glucuronic acid or sulfate.

Previous methods utilized solvent extraction (115,117) to isolate the phenolic metabolites of LID. However, the nature of these phenolic metabolites requires that they be derivatized prior to organic solvent extraction. If underivatized, these amphoteric compounds are poorly extracted from aqueous samples even with appropriate pH adjustment. Several methods have been developed in this study for the derivatization of phenolic amines in aqueous samples (i.e reaction with ethyl chloroformate, acetic anhydride and PFBC). All three reagents readily reacted with phenolic metabolites of LID in an aqueous medium. These metabolites were stable to derivatization with acetic anhydride or PFBC, but, with ethyl chloroformate, significant hydrolysis of the amide bond of 3-OH-LID was observed. The use of ethyl chloroformate as a derivatizing reagent for these compounds was abandoned for this reason.

Acetic anhydride has been used recently to derivatize aminophenols in aqueous samples (302,303). The resulting N- and O- acetyl derivatives could be readily extracted into ethyl acetate. This same approach was utilized for the phenolic amine metabolites of LID. The derivatives formed were efficiently extracted into ethyl acetate, but these derivatives had poor GC characteristics; the observed peak spreading reduced their detectability. Peak spreading has been attributed to adsorption of the compound to the GC column during the chromatographic procedure. In particular, the acetate derivatives of 3- and 4-OH-MEGX were found to severely interact with the GC column (DB-17). MS analysis of the acetylated LID metabolites yielded a number of fragment ions which could be used for identification purposes. However, the mass spectra of positional isomers could not be differentiated. Identification of these chemical isomers also depended upon their GC retention times. Unfortunately, acetate derivatives of isomers were not adequately resolved to permit their conclusive identification.

To improve their detectability, the acetates were further derivatized with TFAA after ammonia hydrolysis of the phenolic acetyl group. This procedure has been applied previously to the low level analysis of other phenolic amine compounds (182), yielding volatile derivatives with improved GC properties. The derivatives formed are sensitive to an ECD. However, either during the ammonia hydrolysis step or more probably during derivatization with TFAA, the amide bond of mono-acetyl-3-OH-LID and di-acetyl-3-OH-MEGX was hydrolyzed. This limited the usefulness of this derivatization procedure.

PFBC has been used for the acylation of phenolic amines in aqueous samples (341). There are several advantages to the use of this derivatizing reagent for GC studies. Not only can the reaction be carried out in an aqueous medium but once formed, these derivatives are easily extracted. The derivatives are also extremely sensitive to an ECD, probably due to the presence of the halogenated phenyl ring (221). Compounds such as amphetamine and tyramine have been analyzed at low levels (1 ng/sample), in a variety of biological samples, using this reagent. All known and novel metabolites of LID could be derivatized with PFBC. The derivatives formed had good GC characteristics and were particularly sensitive to ECD. Using this improved analytical procedure for the detection of the phenolic and dealkylated metabolites of LID, several new metabolites were observed in the urine of rats and humans administered LID. In rats, 3-OH-GX, a methoxylated metabolite 47, 48 (Fig. 76) and the analog of MEGX 49 in which a ring methyl group had been hydroxylated (Fig 77), were all observed following the administration of LID. It is interesting that Fujita et al. (342) identified an analog of LID where one of the ring methyl groups was hydroxylated, in young rat liver perfusion studies. but they did not observe the analagous hydroxylated MEGX metabolite. All the metabolites observed in the current study were identified by EI and CI/MS after derivatization with PFBC. EI fragmentation ions were derived from both the phenolic ring and aliphatic side chain allowing identification of each compound (table 14). The proposed methoxylated analog of MEGX was identified by its EI and CI fragmentation patterns. The EI fragmentation pattern of this compound was identical to that of PFBC-derivatized 3-OH-MEGX except the mass of the molecular ion of this new metabolite was 30 amu higher (Fig 81). The mass of this molecular ion was confirmed using CI/MS. EI fragmentation of the methoxylated metabolite indicated that this compound had an aliphatic side chain similar to PFBC-derivatized 3-OH-MEGX. Present in the mass spectrum of both compounds were the ions m/z 252 and 281 (Fig. 81) (identified in table 14) which are known to be present in compounds structurally related to MEGX. Therefore the side chain of the methoxylated metabolite had to possess the side chain $COCH_2NHCH_2CH_3$ prior to PFBC derivatization.

4-OH-MEGX was identified in the urine of a patient receiving LID (Fig. 78). The identity of this compound was confirmed since it had identical EI/MS, CI/MS and GC retention time to authentic 4-OH-MEGX derivatized with this reagent.

The stability of synthetically prepared 4-OH-LID, 4-OH-MEGX and 4-OH-GX to enzymatic or acid hydrolysis (3N HCl at 100° for 2h) indicated that 4-OH-Xyl, isolated from human urine, was a true metabolite and was not formed during the hydrolysis procedure. To confirm the authenticity of 4-OH-Xyl in human urine the glucuronide conjugate of this compound was isolated from the urine of patients receiving LID and identified by GC/MS. The

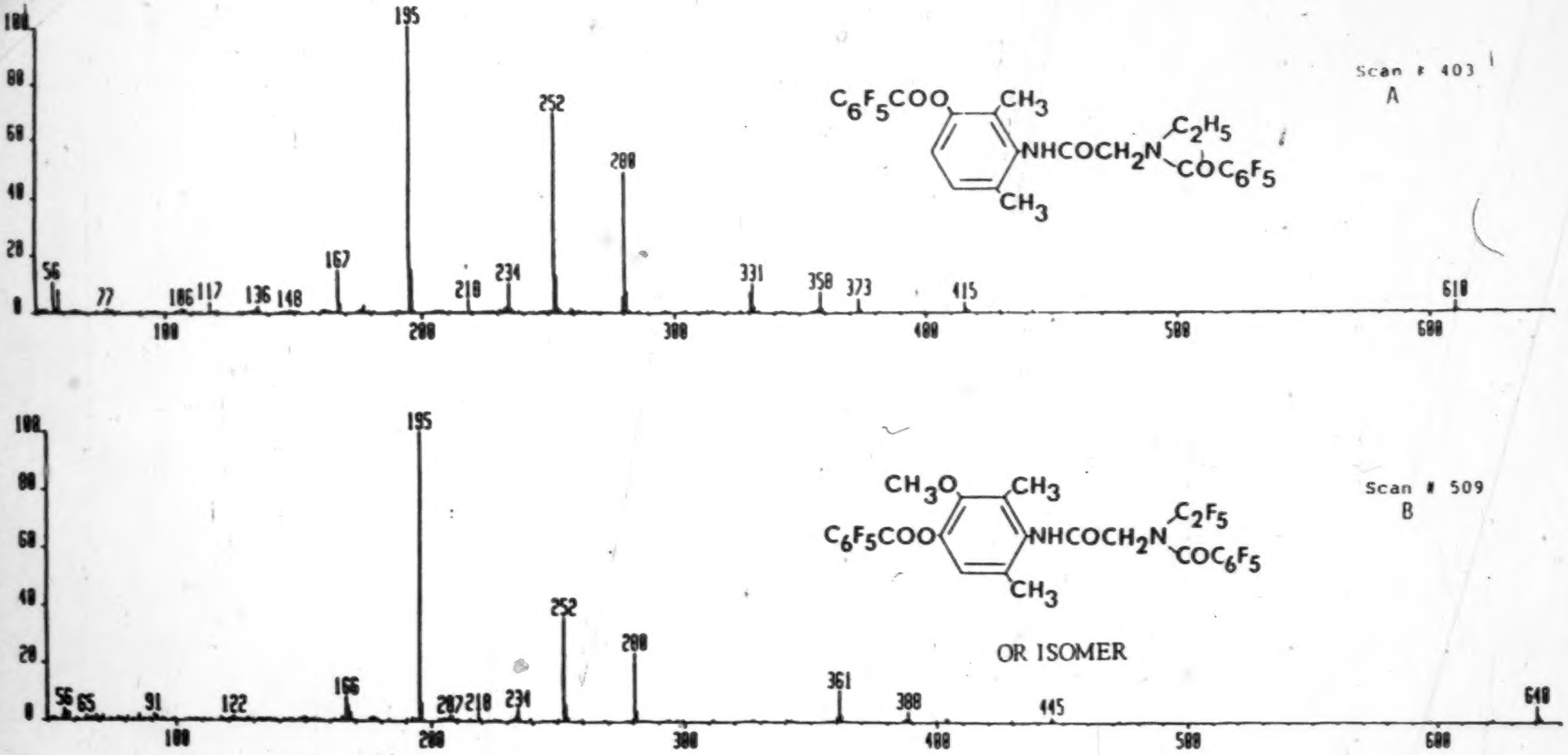


Figure 81. Comparison of the electron-impact mass spectra of PFBC-derivatized 3-OH-MEGX (A) and the proposed methoxylated metabolite, similarly derivatized (B). The structure of the diagnostic fragment ions are identified in table 14.

low volatilities of glucuronide-conjugated compounds requires that they be derivatized prior to GC/MS analysis. Of the available derivatizing procedures, permethylation of the glucuronide conjugate with methyl iodide catalyzed by a strong base (e.g. sulfinylmethide carbanion) was selected (240). This reagent reacts with a variety of functional groups including carboxylic, alcohol and amine functionalities, which permits the derivatization of the conjugate in a single step. Once permethylated the glucuronide conjugate is sufficiently volatile to permit its separation from other components on GC. Other EI/MS studies on several permethylated glucuronide conjugated compounds have demonstrated that these compounds can be identified by their EI and CI/MS fragmentation patterns. Several diagnostic fragment ions derived from the glucuronic acid moiety were present in their mass spectra. With some compounds, fragment ions derived from the aglycone portion of the molecule were also observed. In the present study it was confirmed that the isolation and identification procedures to be used for the identification of 4-OH-Xyl glucuronide were adequate, by subjecting a known glucuronide to the procedure. The known glucuronide of acetaminophen was isolated from the urine of rats administered this drug, permethylated with methyl iodide/sulfinylmethide carbanion and identified by its EI fragmentation pattern. The EI fragmentation pattern observed for this compound was consistent with the published EI fragmentation patterns of related permethylated glucuronides (240). In addition to the fragment ions derived from the glucuronic acid moiety, several fragment ions were present which could be used to identify the aglycone. From all the ions present, the structure of the compound could be deduced. When this same procedure was used to identify the glucuronide of 4-OH-Xyl similar results were observed. Interpretation of its EI fragmentation pattern identified the structure of this metabolite to be the one depicted in figure 66. The primary nitrogen of 4-OH-Xyl glucuronide is dimethylated during the permethylation procedure. Although hydrolysis of the amide bonds of diphenylhydantoin and barbital were observed when permethylated, no hydrolysis is believed to have occurred in this study on acetaminophen, its glucuronide conjugate and 3-OH-LID. All these compounds were found to be stable to this derivatizing reagent. From these results it appears that 4-OH-Xyl is an authentic urinary metabolite of LID.

Although 4-OH-LID, 4-OH-MEGX and 4-OH-GX appeared stable to the analytical procedure used to isolate 4-OH-Xyl it was necessary to ascertain whether these compounds could be substrates for the formation of 4-OH-Xyl *in vivo*. The presence of 4-OH-Xyl in the urine of rats administered LID indicated that these animals do have the capacity to hydrolyze LID or one of its metabolites. Amidase enzymes have been observed in rat liver (343). However, 3-hydroxylation rather than amide hydrolysis takes precedence in the metabolism of LID by these animals. In addition to the 4-hydroxylated compounds, 3-OH-LID, 3-OH-MEGX and Xyl were also administered to rats to determine if these compounds could be converted to 3-OH-Xyl, a metabolite never before identified. 4-OH-MEGX and 4-OH-GX were both good substrates for the amidase enzyme of rats. In fact the insignificant quantities of these compounds observed in the urine of rats given large doses suggests that they are excellent substrates for the enzyme. 4-OH-LID on the other hand could be recovered in the 24 h urine of rats given this compound. In addition 4-OH-Xyl, but not 4-OH-MEGX, was also observed in these urines. The reverse situation was true when 3-OH-LID was administered to rats. In the urine of these animals large quantities of 3-OH-MEGX, but no 3-OH-Xyl was observed. When 3-OH-MEGX was administered to rats, the unaltered drug was simply recovered in the urine. No hydrolysis of this compound was observed. Apparently following the administration of 4-OH-LID, this compound was de-ethylated to 4-OH-MEGX which in turn was rapidly metabolized to 4-OH-Xyl. The difference in the specificity of this enzyme towards 4-OH-LID and 4-OH-MEGX requires further investigation, possibly using a purified amidase enzyme preparations. 3-OH-LID, 3-OH-MEGX and 4-OH-LID all appear to be poor substrates for the amidase enzyme of rats. This offers an explanation as to why 4-OH-Xyl but not 3-OH-Xyl is observed in the urine of rats administered LID. It is conceivable that rats are capable of hydroxylating LID or MEGX in the 3' and 4' positions. However, it is only the 4-OH-LID or 4-OH-MEGX which is further metabolized to 4-OH-Xyl in these animals. In man, a similar situation may exist but this requires confirmation. The observation of 4-OH-MEGX in the urine of a patient treated with LID indicates that man is capable of p-hydroxylating LID, MEGX or both. It is possible that p-OH-LID, p-OH-MEGX or p-OH-GX are the precursors for 4-OH-Xyl and not Xyl as previously suggested.

To confirm that Xyl was a precursor for 4-OH-Xyl, this compound was given to rats. Significant quantities of both 4-OH-Xyl and Xyl were isolated in the 24 h urine of these Xyl treated rats. The ability to convert Xyl to 4-OH-Xyl supports the theory that initially MEGX is converted to Xyl which in turn is p-hydroxylated to form 4-OH-Xyl (115). However, if significant quantities of Xyl were formed from LID one might expect to see methemoglobinemia, a known toxic reaction observed following Xyl administration (344). According to the previously proposed metabolic pathway of LID, approximately 70% of a dose of LID is initially converted to Xyl, which is then hydroxylated to form 4-OH-Xyl. Yet, methemoglobinemia following LID administration has only been observed when other compounds, such as phenacetin are administered at the same time as LID. The absence of methemoglobinemia following LID administration implies that the amount of Xyl actually formed from a dose of LID is very small. It is conceivable that a large quantity of 4-OH-Xyl is derived from the p-hydroxylated analogs of LID, MEGX or GX.

3.2.3 Conclusion

From these studies in the rat it is apparent the 4-hydroxylated analogs of LID, MEGX and GX are metabolized to 4-OH-Xyl. The conversion of 4-OH-LID to 4-OH-Xyl is believed to proceed through 4-OH-MEGX. These compounds appear to be far better substrates for the amidase enzyme of rats than are the corresponding 3-hydroxylated isomers, suggesting that this enzyme displays substrate specificity. Previously it has been demonstrated that by replacing the chlorine atom of butilicaine with a methyl group the amidase activity toward this compound was reduced by 75% (343).

The results from these studies imply that in man significant quantities of 4-hydroxylated LID, MEGX, or GX may be formed as intermediates in the formation of

4-OH-Xyl. Using the pathway of LID metabolism proposed in this study, competition for the hydroxylation pathway may result in the saturation of the enzyme resulting in the observed LID kinetics following i.v infusion. It would be important to determine if any of these hydroxylated metabolites (4-OH-LID, 4-OH-MEGX, 4-OH-GX) can inhibit either the hydroxylation or the N-dealkylation pathways. This would help explain the time-dependant kinetics of LID observed during i.v. infusion.

3.3 Studies Conducted on Doxapram

3.3.1 Analysis

Currently DOX is being evaluated (36) as a respiratory stimulant in premature infants. However, most of the knowledge of the pharmacokinetics of this drug has been derived from studies conducted in adults (40). The inability to determine the pharmacokinetics in premature infants is in part due to the lack of appropriate analytical techniques used for the analysis of DOX and its metabolites in these patients. The method used must be extremely sensitive since only small samples of blood may be withdrawn from these patients.

Current methods available for the analysis of DOX include HPLC (121), GC (40,45) and U.V. spectrophotometry (91). Of these methods, only GC linked to either a NPD or MS possessed the sensitivity and specificity required for the analysis of DOX in biological samples. An HPLC method has been described for the analysis of DOX metabolites but this procedure lacked the sensitivity needed to detect unchanged DOX in dogs administered this compound. Current GC methods used for the analysis of DOX and its major metabolites could not be applied to the low level analysis of these compounds Poor peak resolution observed when these compounds were co-chromatographed reduced the sensitivity of the procedure (45). The peak spreading observed may be due to the interaction of DOX or its metabolites with the column (i.e. glass surface) during the chromatographic procedure. Adsorption of DOX to the surface of glass has been observed in this study when an aqueous sample of DOX was stored, overnight, in a glass vial. Appropriate column selection did improve the chromatographic separation of DOX and the metabolites 1-ethyl-4-(2-ethylamine)-3,3-diphenyl-2-pyrrolidinone (compound b, Fig. 3) and 1-ethyl-4-(2-hydroxyethyl-aminoethyl)-3,3-diphenyl- 2-pyrrolidinone 11. However, low level analysis could only be obtained using GC/MS-SIM (45). In this study a new GC-NPD procedure was developed using a fused silica capillary column to overcome the limitations observed with previous methods used for the analysis of DOX.

Highlights of this procedure are a) performing the extraction procedure in plastic 1.5 ml microfuge tubes and b) improved separation and resolution of the compounds using a 9 m DB-5 capillary column. Once separated the compounds were detected, following their elution from the GC column, using a NPD. By performing the extraction procedure in plastic vials, the recovery of DOX was such that even after a single extraction step (5 min vortex followed by 5 min centrifugation) enough DOX was recovered to permit detection of 1 ng of material per aqueous sample. Capillary columns provide better resolution of the chromatographic peaks, since these columns have an increased number of theoretical plates compared to 3mm i.d. glass GC columns packed with stationary phase coated solid support (e.g. chromosorb W) material. The improved resolution obtained with capillary columns increased the sensitivity and accuracy of the analytical procedure. Further improvements were made by selecting an appropriate stationary phase. When DB-17 (J&W Scientific) and DB-1 (J&W Scientific) stationary phases were tried, excessively high temperatures were required for the elution of DOX. These two phases were inappropriate for the analysis of DOX. A DB-5 stationary phase proved to be the only appropriate stationary phase for the low level analysis of DOX. The technique developed allowed for the analysis of DOX in successive plasma samples (20-100 ul) obtained from premature infants. The assay procedure was rapid, requiring no additonal clean-up procedures other than protein precipitation with perchloric acid. To construct calibration graphs, blank plasma samples (50 ul) spiked with a known quantity (0-5 ug) of DOX and a standard quantity of diazepam (I.S.) were analyzed using this procedure. The actual graphs were generated by plotting the ratio, obtained by dividing the integrated GC peak area of DOX by the GC peak area of the internal standard, against the corresponding concentration of DOX in the sample. Calibration graphs constructed in this manner were linear over the concentration range 0-5 ug. The slope of the standard curves, generated in this manner, on different days varied from 0.405 to 0.413. The coefficient of correlation calculated for these standard curves were consistently above 0.998. The coefficient of variance was found to be 5.3% when triplicate samples of blank plasma spiked with DOX (2.5 ng/50 ul plasma) were analyzed using this analytical procedure. Typical GC traces of the solvent extract of urine and plasma obtained from premature infants infused with DOX are depicted in figure 82. Included are plasma and urine blanks, which contained no DOX, carried through the extraction procedure.

3.3.2 Metabolism and Pharmacokinetics of Doxapram

The identity of the GC peak with the retention time 7.14 min (Fig. 82) was deduced to be metabolite 11 by interpretation of its EI fragmentation pattern (Fig 83). This metabolite has only been observed in the urine of premature infants and dogs, but not adults. The morpholinone metabolite 12, observed in the plasma of adults, was not observed in these premature infants. The same observation has been made by others (28) and suggests that premature infants and adults metabolize DOX differently. It is apparent that adults and infants initially oxidize DOX to form the α-hydroxyamine compound. In adults, this compound is further oxidized to the morpholinone metabolite 12 (Fig 84). However, it appears that infants are not capable of carrying out this last oxidation to form the lactam. Since α-hydroxy amine compounds are unstable (345), opening of the morpholine ring occurs, forming the corresponding aldehyde. Although this metabolite has never been identified in the urine or plasma of premature infants, this intermediate has been tentatively identified in the urine of dogs (122). This aldehyde undergoes a second oxidation, followed by the loss of $OCHCH_2OH$, to form 1-ethyl-4-(2-hydroxyethylaminoethyl)-3,3-diphenyl- 2-pyrrolidinone (Fig. 84).

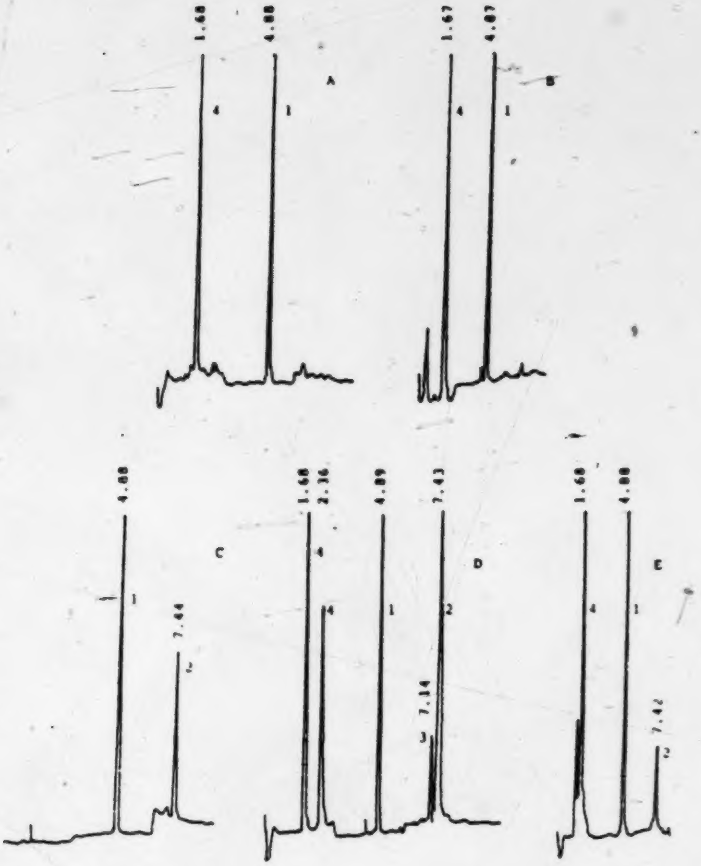
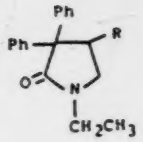


Figure 82. GC traces of A) blank plasma B) blank urine; C) patient plasma sample (50 ul); D) patient urine sample (30 ul); E) plasma sample (100 ul) spiked with 2.5 ng doxapram. The patients were premature infants who were receiving doxapram by i.v. infusion (1-2.5 mg/kg/h). The peaks are identified as 1= diazepam; 2= doxapram; 3=doxapram metabolite(XI); 4=endogenous components. The retention times are shown in minutes.



m/z	R
352	$-CH_2CH_2\overset{+\cdot}{N}HCH_2CH_2OH$
334	$-CH_2CH_2\overset{\cdot\cdot}{N}HCH=CH_2$
321	$-CH_2CH_2\overset{+}{N}H=CH_2$
291	$-CH=CH_2]^{+}$
278	$-\overset{+}{C}H_2$

Figure 83. The electron impact fragmentation pattern of the doxapram metabolite 11 isolated from the urine of premature infants receiving doxapram.

Changes in the metabolism of DOX in premature infants will be associated with changes in the pharmacokinetics of this drug in this population. The assay developed in this study was used to determine the ClTB, Clr and T1/2 of DOX in premature infants (Table 15). ClTB and Clr were calculated during steady state infusion of the drug (Appendix C and D). Both the ClTB (8.87 ± 2.7 ml/min) and Clr (0.41 ± 0.19 ml/min) values were significantly reduced from those observed in healthy adults. The T1/2 of DOX in these infants (10.04 h) was almost 3 times as long as observed for adults (3.4 h). The results obtained for ClTB and T1/2 in this study agreed with those values previously found. However, no correlation between age and ClTB was observed (28). Data obtained from a previous study suggested that a correlation may exist.

Often the ClTB of a drug is reduced from adult values when administered to infants. Infants and small children do have a reduced capacity to metabolize drugs (54). Some metabolic pathways, such as glucuronide conjugation, are not functional until well into the first year of life (54). Since DOX is extensively metabolized in man it appears that the decrease in the ClTB observed in premature infants may be associated with changes in the metabolism of this drug. In part this could be due to an impairment of the oxidation of DOX to the morpholinone metabolite 12. The ability to oxidize the α-hydroxy amine 50 to the morpholinone metabolite 12 appears to be impaired in these infants. The net result of reduced metabolism is that higher blood levels of DOX will be observed since elimination of this compound is predominantly by the non-renal route (i.e. metabolism). To obtain therapeutic blood levels of DOX in these infants would require the infusion of less drug on a per kg basis than would have to be administered to adults.

3.3.3 Discussion

Once the pharmacokinetic parameters of DOX in premature infants are established, improvements in the administration of this drug can be made. Currently the dose of DOX administered to these infants is based on pharmacokinetic information gained from studies

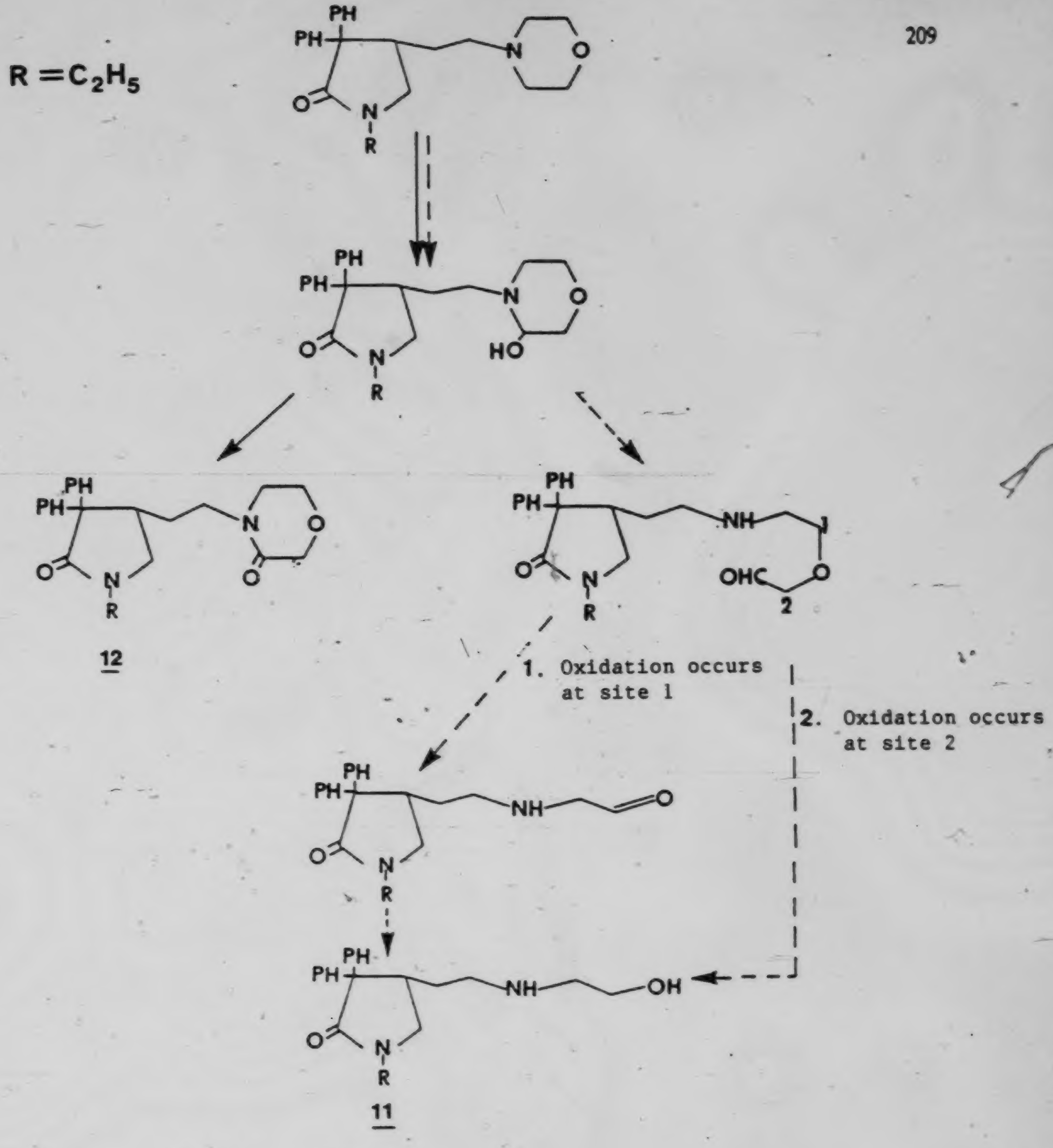


Figure 84. The proposed pathways of doxapram metabolism in adults and premature infants.
Pathway reported in adults ——
Possible pathway in premature infants - - - -

Table 15. The pharmacokinetic parameters, total body clearance (Cl_{TB}), renal excretion rate dXu/dt, renal clearance (Cl_r) and half-life (T1/2) calculated for doxapram administered to premature infants. The experimentally determined plasma concentration of doxapram in these infants is also listed as Co and C6. Other parameters listed in this table are weight (Wt.), creatinine clearance (Cl_{cr}) and the doxapram infusion rate (Ko).

Patient	Wt	Cl_{cr}	Ko[1]	Co[2]	C6[2]	Css[2]	Cl_{TB}[3]	dXu/dt[4]	Cl_r[5]	T1/2[6]
Pa	1.33	79	2.5	7.39	10.1	8.74	6.20	2.06	0.654	-
Gr	1.40	60	2.0	4.18	5.3	4.74	9.84	4.72	2.770	-
Pe	1.00	47	2.5	2.65	4.61	3.63	11.48	0.752	0.575	-
			1.25	3.02	1.27	2.14	9.73	0.342	0.444	-
Ru	0.88	68	2.0	4.96	3.50	4.20	6.98	0.56	0.37	13.9
Gr	0.9	64	1.0	1.04	1.52	1.28	11.72	0.13	0.282	11.2
			2.0	5.06	4.60	4.83	6.22	0.25	0.145	-
Ml	0.81	-	2.0	1.33	-	-	-	-	-	9.9
Og	1.09	68		N.D	N.D	N.D.	N.D.	N.D.	N.D.	8.45
WaA	0.90			N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	9.12
Hl	1.05			N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	9.36
La	0.90	52		N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	6.9
WaB	1.16			N.D.	N.D.	N.D.	N.D.	N.D.	N D	13.1
Mean							8.9		0.75	10.24
±S.D.							2.4		0.91	2.4

1. mg/kg/hr
2. ug/ml plasma
3. ml/min
4. mg/6 h
5. ml/min
6. h

Co= the concentration of DOX in the first plasma sample withdrawn during steady state.

C6= the concentration of DOX in the second plasma sample withdrawn, during steady state, 6 hours after the first plasma sample.

conducted in adults. Since these infants metabolize DOX more slowly, this could lead to excessively high blood levels and the possibility of a toxic response. A second drawback to the current method of DOX administration is that plasma steady state levels are not attained for almost 50 h after i.v. infusion of this drug is initiated. Often therapy is discontinued if no beneficial response is observed within 24 h, well before steady state is attained. The administration of a loading dose prior to i.v. infusion is often used to quickly bring the plasma levels of DOX to steady state. It would be of benefit in the treatment of these infants to determine an appropriate DOX loading dose. This would ensure that therapeutic blood levels are rapidly attained, following the start of therapy. To further this study it is essential to determine the plasma level of DOX required before beneficial therapeutic response is observed in these infants. Once determined, an appropriate loading dose and i.v. infusion can be administered safely and effectively.

4. Conclusion

During the course of this study several new analytical procedures were developed for the analysis of PLZ, LID and DOX and several of their respective metabolites. In most instances GC was selected due to the high sensitivity and resolving power of this analytical method. An added advantage of GC is the relative ease with which the chromatograph can be linked to a MS for the unequivocal identification of the compounds of interest and any new metabolites which may be present in the biological sample.

All the analytical procedures in this study were sensitive, specific and reproducible. Confirmation of the compounds or derivatives (formed during the analytical procedure) was done by interpreting each EI and CI mass spectrum and comparing it to authentic standards similarly prepared. Once developed, these techniques were used to analyze for these drugs and their metabolites in a variety of biological fluids (e.g. blood, urine, and bile).

PLZ was measured in blood and bile samples obtained from rats following its administration to these animals. Besides the conversion of PLZ to PAA and PEA, little else was known about the metabolism of this drug. In this study a new *in vivo* metabolite, EBZ, was isolated from the expired air of PLZ-treated rats and identified by GC/MS.

In addition to metabolism, a significant quantity of PLZ was observed in the bile of rats treated with this drug. Biliary excretion is a saturable process and could in part explain the increase in T1/2 observed with chronic PLZ administration. It is conceivable that the biliary excretion of PLZ becomes more pronounced with chronic PLZ dosing since only 40% of a PLZ dose could be recovered in urine and expired air after 24 h.

With chronic PLZ administration, the percent of a PLZ dose converted to EBZ or PEA increased slightly. This is an important observation since both metabolites are pharmacologically active *in vivo*. EBZ is a product of PLZ formed through a free radical intermediate. This free radical intermediate can covalently bind to a number of tissue components causing tissue damage. PEA on the other hand has amphetamine-like stimulatory properties, which could be beneficial in antidepressant therapy. The delay in the accumulation of PEA could correlate with the delay observed in the clinical response to PLZ treatment. Whether similar results occur in humans has yet to be determined.

With the development of a superior analytical technique for the analysis of the amine and phenolic amine metabolites of LID, investigations into the metabolism of this drug were initiated. The analytical procedure offered several advantages over previously used methods; a) quantitative recovery of the phenolic amine metabolites, b) production of a derivative which permitted low level analysis of these compounds and c) separation of all ten known and proposed metabolites of LID. With this analytical method, four new metabolites of LID were discovered. Three of these metabolites were observed in the urine of rats administered LID while 4-OH-MEGX was observed in the urine of a patient receiving an i.v. infusion of LID. It is obvious from this study that the metabolism of LID is far more complicated than previously thought.

The observation that 4-OH-MEGX was present in human urine supports the pathway of LID metabolism, in man, proposed in this study. This pathway suggested that LID or MEGX were initially converted to their p-hydroxylated analogs, which in turn are metabolized to 4-OH-Xyl. In rats 4-OH-MEGX and 4-OH-GX were good substrates for the amidase enzyme, *in vivo*.

Investigations into the metabolism of LID were initiated to help explain the dose- and time-dependant kinetics observed when this drug is administered to patients. In rats a similar dose- and time-dependant kinetics of LID metabolism has been observed. However, in this animal these kinetics were attributed to saturation of the 3-hydroxylation pathway. In man, however, this explanation could not be used because it was thought that aromatic hydroxylation of LID or one of the de-ethylated metabolites (except XYL) did not occur. However, using the pathway proposed in this study it is apparent that a mechanism similar to the one used to explain LID kinetics in rats could be used to explain the kinetics observed in man.

The metabolism and pharmacokinetics of DOX were evaluated in premature infants administered this drug. Essential for this study was the development of a highly specific and sensitive analytical procedure for the analysis of DOX in the plasma of premature infants. A sensitive procedure was necessary since only small volumes of plasma could be withdrawn from these infants. The procedure developed here enabled accurate estimation of the T1/2 of DOX since this drug could be accurately measure in five consecutive plasma samples, withdrawn 2 h apart, once infusion was terminated. It is evident from these investigations that these infants have a prolonged T1/2 and reduced ClTB. In light of these observations it is essential that premature infants be given a reduced dose of DOX.

It is also evident from the long DOX T1/2 observed in these infants that steady state plasma levels of DOX would not be reached until nearly 50 h. To overcome this long delay before steady state is reached it would be pertinent to establish and administer a DOX loading dose to these infants. Not only would a steady state blood level of DOX be rapidly attained but the therapeutic response to DOX would also be more rapid.

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